GENETICS

A periodical record of investigations bearing on heredity and variation

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PUBLISHED MONTHLY AT AUSTIN, TEXAS
BY GENETICS, INC.
(Date of issue, January 30, 1962)

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VOLUME 46 DECEMBER, 1961 NUMBER 12

GENETICS is a monthly journal issued in annual volumes of about 1000 pages each.

Subscription, $12.00 net a year for complete volumes (January–December). Foreign postage, $1.00 additional. Single copies, $1.50 each, postpaid. GENETICS is sold by volume only. All subscriptions must begin with the January issue.

As available, back volumes 1 through 44 may be had at $14.00 each and Volume 45 may be purchased for $28.00. Single issues of previous volumes are $2.50 per copy, postpaid. The Business Office will supply information on request as to volumes and numbers available.

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Second-class postage paid at Austin, Texas.

Claims for missing numbers should be made within 30 days following their date of mailing. The publishers will supply missing numbers free only when they have been lost in the mails.

COMPARISON OF EFFECTS OF ATOM DECAY AND BETA-RAY RADIATIONS ON THE INACTIVATION AND MUTATION OF A MYCOBACTERIUM

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Received June 14, 1961

INACTIVATION of bacteria by incorporated radioactive phosphorus (P^{32}) was reported by FUERST and STENT (1956) and KAUDEWITZ, VIELMETTER and FRIEDRICH-FREKSA (1958). It is not always easy to differentiate the effect of P^{32} decay from the effect of intracellular beta-ray irradiation, although differentiation from the effect of extracellular radiations could have been made by these authors. The present author (TSUKAMURA 1960a) previously made a comparative study on the mutagenic effects of P^{32} and S^{35} (radioactive sulfur) incorporated into cells of a Mycobacterium. Since, under the conditions tested, intracellular radiations with S^{35} could cause neither inactivation nor mutation in cells, it was suggested that decay of P^{32} atoms plays an important role in causing inactivation and mutation. Thereafter, some additional experiments were made (TSUKAMURA 1961). The organism was labeled with P^{32}-orthophosphate under different conditions, and the relationship between the intracellular distribution of P^{32} and inactivation and mutations in these cells was followed. The results revealed that increase of P^{32} incorporation into the deoxyribonucleic acid (DNA) fraction caused an increase of inactviation and mutation, and, in addition, an inactivation frequency that fitted a curve of a single-hit-type was found in cells containing a higher amount of P^{32} in their DNA fraction. It was considered thus that decay of P^{32} in the DNA mainly causes inactivation and mutation. However, inactivation and mutation were also caused but to a less extent in cells containing a large amount of P^{32} outside the DNA fraction and a slight amount of it in the DNA fraction. In the latter case, the possibility could not be excluded that intracellular beta-ray radiations may have taken a part in causing inactivation and mutation, although it was probable that, as well, accumulation of decays in cytoplasm is associated with such effects considering the occurrence of inactivation curves of a multihit type.

The present study was designed to determine what role is played by the beta-ray radiations that always accompany the decay phenomenon. For this purpose, the effect of radioactive strontium (Sr^{90}) having a very long half decay period (19.9 years) and a strong beta-ray radiation capacity (E_{max} 0.65 Mev, 2.35 Mev) was compared with the effect of P^{32} (half decay period 14.3 days, E_{max} 1.71 Mev).

MATERIALS AND METHODS

An avirulent strain Jucho of *Mycobacterium avium* was used. The organism was subcultured in 50 ml of Sauton medium (in 200 ml Erlenmeyer flasks) to

which were added P^{32} and Sr^{90}. Inoculation was made with 3 mg (wet weight) of cells per flask. The media inoculated were incubated at 37°C for six days and the resulting cultures were used for determining viable counts and mutation frequencies to streptomycin resistance and to isoniazid resistance. P^{32}-orthophosphate (specific activity CF, Radiochemical Center, Amersham, England) and Sr^{90}-nitrate (specific activity CF, Oak Ridge National Laboratory, Oak Ridge, Tennessee, U.S.A.) were used for the experiments.

Methods for determinations of the number of viable cells per mg (wet weight) and of the mutation frequencies were described previously (TSUKAMURA 1960a). Counts were made after five days of incubation and dilutions giving rise to 20 to 200 colonies per plate were used for the basis of calculation.

Supernatants of the cultures were obtained by centrifugation and, after drying, their radioactivities were counted by a Geiger-Mueller counter (count efficiency for P^{32} 5%). Cells of the cultures were washed five times in distilled water, measured for their wet weight and counted for their radioactivities. Radioactivity was expressed as counts per minute (cpm). The appropriate corrections for background and natural decay were applied.

RESULTS AND DISCUSSION

Growth: The results are shown in Table 1. In media containing different concentrations of isotopes and containing no radioisotope, almost similar amounts of growth were observed, although a culture growing in a medium containing 20 μc Sr^{90}/ml exhibited somewhat delayed growth during the first few days. Amounts of growth as measured after six days of incubation did not differ significantly (Table 1). It appears, therefore, that the growth phases of these cultures do not differ markedly (this organism enters the stationary growth phase after three days (TSUKAMURA 1960b)).

Viability of cells: The results are shown in Table 2. In number of viable cells per mg only the culture grown in medium containing 1 μc P^{32}/ml showed a marked decrease, to about one sixth of that for the control culture grown in medium containing no radioisotope. However, no decrease occurred in the culture grown in medium containing 20 μc Sr^{90}/ml, which was exposed to much more intracellular and extracellular beta-ray radiations than was true for the culture grown in medium containing 1 μc P^{32}/ml (Table 3).

TABLE 1

Amount of growth occurring after six days of incubation

Medium		Amount of growth per flask
Control (no radioisotope)		1.8 gm (wet weight)
Sr^{90}-nitrate	2 μc/ml	1.9
Sr^{90}-nitrate	20 μc/ml	1.7
P^{32}-orthophosphate	1 μc/ml	1.6

Each medium was inoculated with 3 mg (wet weight) of cells.

TABLE 2

Viability and mutation frequencies in cultures grown in media containing no radioisotope and in media containing radioisotopes in concentrations of 2 μc Sr^{90}/ml, 20 μc Sr^{90}/ml and/or 1 μc P^{32}/ml

Medium	No. of viable cells per mg (wet weight)	Ratio of INH-resistant mutants* per 10^8 viable cells‡	Ratio of SM-resistant mutants† per 10^8 viable cells‡
Control	$(2.89 \pm 1.43) \times 10^6$/mg	19.8±12.4	2.91± 0.550
Sr^{90} 2 μc/ml	$(1.91 \pm 1.14) \times 10^6$/mg	11.9± 4.04	4.55± 2.33
Sr^{90} 20 μc/ml	$(3.25 \pm 1.73) \times 10^6$/mg	67.7±44.3	10.8 ± 5.38
P^{32} 1 μc/ml	$(4.40 \pm 2.95) \times 10^5$/mg	174. ±45.0	89.2 ±41.0

* Mutants resistant to 10 μg isoniazid/ml.
† Mutants resistant to 1 μg streptomycin/ml.
‡ Mean and standard deviation obtained by three independent determinations on the same culture. In each determination, the number of viable cells was calculated as an average of five plates. The relatively high deviations in the data seem to have resulted from doubling of the errors involved in estimations of the number of viable cells and the amount of cells (mg) or the errors involved in calculations of the number of total viable cells and the number of mutants.

TABLE 3

Radioactivities of culture filtrates and of bacterial cells

Medium	Radioactivity of culture filtrate	Radioactivity of bacterial cells
Control	0. cpm/0.2 ml	0. cpm/mg (wet weight)
Sr^{90} 2 μc/ml	38,265.	1,397.
Sr^{90} 20 μc/ml	380,200.	11,425.
P^{32} 1 μc/ml	16,332.*	1,781.*

cpm = counts per minute.
* Corrected into cpm at the beginning of incubation.

In view of the results, it is concluded that inactivation of cells occurring in the culture to which 1 μc P^{32}/ml was added is not due to intracellular beta-ray radiations but due to decay of P^{32} atoms.

Mutation frequencies: The highest mutation frequencies were found in the culture to which 1 μc P^{32}/ml was added, and a slight increase in mutation frequency was seen in the culture given 20 μc Sr^{90}/ml. No significant increase was seen in the culture receiving 2 μc Sr^{90}/ml, which would have been irradiated by about the same amount of intracellular beta-ray radiations and by a much greater amount of extracellular radiations than was true for the culture getting 1 μc P^{32}/ml.

In view of these results, it is conceivable that increase of mutation frequencies occurring in medium containing 1 μc P^{32}/ml is mainly the result of decay of P^{32} and is not due to the beta-ray radiations whether they were intracellular or extracellular. It also seems probable that the increase in mutation frequency, which was observed previously (TSUKAMURA 1961) in cells whose DNA fraction was labeled with only a little amount of P^{32}, was due to decay of P^{32} atoms in the cytoplasm. On the other hand, a large amount of Sr^{90} (i.e., beta-ray radiations) could cause mutations, since the effect of decay is negligible in this iso-

tope. It appears remarkable thus that in contrast to the effect of P^{32}, Sr^{90} is mutagenic in a large amount without inactivation.

SUMMARY

Effects of P^{32} and Sr^{90} added to culture media were compared in a Mycobacterium to determine whether decay of atoms or intracellular beta-ray radiations takes an important role in causing inactivation and mutation. Both inactivation and mutation occurred significantly in a culture grown in medium containing 1 μc P^{32}/ml, and only a moderate increase of mutations occurred without inactivation of cells in a culture grown in medium containing 20 μc Sr^{90}/ml. No significant increase of mutation was seen in a culture grown in medium containing 2 μc Sr^{90}/ml.

Since large amounts of intracellular and extracellular beta-ray radiations from Sr^{90} were found to be ineffective in causing inactivation of cells and much less effective in causing mutation than is true for a small amount of P^{32}, it is conceivable that inactivation and mutations occurring in cells grown in medium containing such a small amount of P^{32} (i.e., 1 μc P^{32}/ml) are due neither to intracellar nor extracellular beta-ray radiations but mostly to decay of P^{32} atoms incorporated into cells.

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ESTIMATE OF THE LOAD OF MUTATIONS IN HOMOGENEOUS POPULATIONS FROM DATA ON MIXED SAMPLES[1]

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Received October 13, 1961

THE load of mutations estimated for a heterogeneous (from the ethnic point of view) sample may be used for calculating the same load in one of the "homogeneous" populations which contributed to form the mixed sample, provided one also knows the load of the other "homogeneous" group. The method has been developed as follows:

$$M_{he} = M_{ho}g_{ho} + M_x g_x,$$

where M_{he} is the load in the "heterogeneous" sample, M_{ho} is that of the "homogeneous" group contributing with a proportion g_{ho} of genes to the pooled sample, and M_x is the unknown load of the other "homogeneous" group contributing with a proportion g_x of genes to the same pooled sample.

As $g_x = 1 - g_{ho}$,

$$M_{he} = M_{ho}g_{ho} + M_x(1 - g_{ho}),$$
$$M_x(1 - g_{ho}) = M_{he} - M_{ho}g_{ho}$$
$$\text{and } M_x = \frac{M_{he} - M_{ho}g_{ho}}{1 - g_{ho}}$$

For complex situations involving more than two "homogeneous" groups contributing to a highly "heterogeneous" pooled sample, similar formulae may be easily derived on the same reasoning.

The value of g_{ho} in the above formulae may be roughly estimated in cases involving Whites and Negroes on the basis of information on the general appearance of the individuals concerned. In such instances, $g_{ho} = \frac{\Sigma\, wn}{N}$, where w is the mean fraction, in a given group of n individuals defined according to general appearance, of genes coming from the *ho* population, and N is the total number of individuals in the *he* sample. Therefore,

$$M_x = \frac{M_{he} - M_{ho}\dfrac{\Sigma\, wn}{N}}{1 - \dfrac{\Sigma\, wn}{N}}$$

$$M_x = \frac{NM_{he} - M_{ho}\,\Sigma\, wn}{N - \Sigma\, wn}$$

[1] This research was supported by grants from CAPES, the Rockefeller Foundation, the National Nuclear Energy Commission of Brazil, the National Research Council of Brazil, and the Research Council of the University of Paraná.

This formula has been applied to the data of FREIRE-MAIA (1961), where the mean number M_{ho} of lethal equivalents per White individual was estimated as 0.81 ± 0.86; the same parameter M_{he} in a sample containing 39 Negroes, 15 dark Mulattoes and 26 light Mulattoes ($N = 80$) was found to be 8.97 ± 2.20; and the fraction w of "White" genes in the groups of light and dark Mulattoes was assumed to lie respectively between 0.60–0.90 and 0.10–0.40. The value M_x estimated for Negroes is between 11.19 and 13.71, that is, roughly around 12. A previous direct estimate led to 10.21 ± 4.93 (FREIRE-MAIA 1961, based on data of FREIRE-MAIA, FREIRE-MAIA and QUELCE-SALGADO 1961) .

Data on inbreeding effect reflect the gene pool of the generation of the ancestors common to the related spouses. Therefore, for estimates like the above, it would be necessary to have the ethnic composition of those ancestors and not that of the spouses. The frequencies obtained within the group of spouses may be accepted only as an approximation which may not reflect with accuracy the status among the ancestors. As it is known that the inbreeding levels are, and seem to have been, higher among Whites than non-Whites (FREIRE-MAIA 1960, 1961; FREIRE-MAIA *et al.* 1961), it is possible that the frequency of "White" genes in the non-White consanguineous group may be higher than that estimated on the basis of the phenotype of the spouses.

SUMMARY

A formula has been presented for estimating the load of mutations in "homogeneous" populations from data on "heterogeneous" ones. It has been applied to the situation among Brazilian non-Whites to obtain the value for Negroes. This indirect procedure led to an estimate of about 12 lethal equivalents per individual. This figure agrees with a previous direct estimate of 10.21 ± 4.93.

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THE USE OF INTERCHANGES INVOLVING CHROMOSOME 6 IN MAIZE IN STUDIES OF CHROMOSOME SEGREGATION

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First received October 7, 1960

CHROMOSOMAL interchanges are exchanges of pieces between two non-homologous chromosomes. The exchanged pieces when they pair with their similar parts of the normal chromosomes during meiosis from a configuration of four chromosomes as a "cross" in pachytene stages and as a ring in diakinesis.

Following 2–2 segregation, alternate or adjacent chromosomes may pass to the same pole at the end of division I. When there is no crossing over in the interstitial segment (the part of the chromosome between the centromere and the interchange break point), in alternate segregation the two interchanged chromosomes pass to one pole and complement each other. The same is true for the two normal homologues that pass to the other pole. Consequently the spores formed are fertile.

McCLINTOCK (1934, 1945) has recognized two types of adjacents: adjacent-1 in which homologous centromeres pass to opposite poles, and adjacent-2 in which they pass to the same pole. The four spores that result in each case are abortive. All carry a deficiency and a duplication. When crossing over occurs in an interstitial segment, alternate and adjacent-1 segregations produce spore quartets with two fertile spores that have normal chromosome complements (one with the two interchanged chromosomes, and one with the two normal homologues) and two abortive spores that have deficiency and duplication.

Chromosome 6 in maize has been found to carry a nucleolar organizer region and in meiosis remains associated with the nucleolus until its disappearance at the prophase. Then the nucleolar organizer regions of the split chromosomes form new nucleoli in the spores at the end of division II.

The type of chromosome segregation can be determined by studying the nucleolar make-up of the spores which remain in quartets for a time after the completion of meiosis. Spores which have received no nucleolar organizer region have scattered or diffuse nucleoli. As described by BURNHAM (1949, 1950), spore quartets that arise from adjacent-2 segregation when there is no crossing over in the interstitial segment are expected to have two spores with diffuse nucleoli, and two with potentially two nucleoli that may fuse. Spore quartets that arise from alternate or adjacent-1 segregations following crossing over in an interstitial segment have two spores with one nucleolus, one with diffuse and one with potentially two nucleoli. These latter two spores are the ones that abort; the former two are the fertile combinations. One important difference is that when alternate segregation

follows such crossing over, the crossovers are in the abortive spores whereas when adjacent-1 segregation follows such crossing over, the crossovers are in the fertile spores. (For diagrams, see BURNHAM 1950). A diploid species that has predominantly alternate segregation in chromosome rings will show some spore abortion, the amount being dependent on the frequency of crossing over in the interstitial segments.

Hence three types of spore quartets may be recognized cytologically; the normal type with no spores with diffuse nucleoli, and the two abnormal types, one with one diffuse and one with two diffuse.

In determining the mode of segregation in maize, BURNHAM (1950) has used 27 interchange heterozygotes that had one of their breaks at different loci of chromosome 6. He has divided them into three main groups as follows: the first group with the break in the short arm between the centromere and the nucleolar organizer; the second with the break in the long arm or in the satellite; (In each of the above groups some had short interstitial segments, others had at least one of the two long.) and the third with the break in the nucleolar organizer was not used in his studies.

MATERIAL AND METHODS

In 1951, BURNHAM, E. G. ANDERSON and LONGLEY provided the writer with seeds of 40 other interchange heterozygotes between chromosome 6 and the arms of all the other nine chromosomes. They were planted in the greenhouses at the California Institute of Technology. The microsporocytes were collected and the cytological study of them was carried on in the laboratory of DR. A. E. LONGLEY in the Institute.

The acetocarmine smear method was used in determining the breakage points of the interchanged chromosomes in the pachytene stage and the nucleolar make-up of the spore quartets.

EXPERIMENTAL RESULTS AND DISCUSSION

The breakage points of the interchanges involved in this study have been located and the interstitial segments were measured. In this study, the interchanges in group 2 varied in length of interstitial segments from short to long, whereas in the previous study only the extremes were used. The interchanges were classified according to the breakages in chromosome 6 into three main groups: (1) break in short arm of 6, (2) break in long arm of 6 and (3) break in satellite. The spore quartet data for each group were recorded as indicated in Table 1.

In the first group, as indicated in Table 1, the proportion of "two diffuse" quartets did not exceed 25 percent. There was a negative correlation between the percentages of "two diffuse" type and the total length of the interstitial segments as measured by the percentages of "one diffuse" quartets. That means, the lower the frequency of crossing over in the interstitial segment, the higher the frequency of alternate and probably of adjacent-2 segregations. The latter was not

TABLE 1

The ANDERSON *identification numbers, break positions in the chromosomes, total lengths of the interstitial segments, and percentage of the "two diffuse" and "one diffuse" type of spore quartets in plants heterozygous for interchanges with the break in chromosome 6 in the short arm (first group)*

Interchange	File no.	ANDERSON* A.R.S. 34–4 no.	Break in S6	Break in L of other chromosome	Total length of interstitial segments in μ	Percent of two diffuse	Percent of one diffuse
6–10	30–69	5519	.75	.17	13.54	21.6	6.6
3–6	36–121	6566	.35†	.41	21.81	12.6	67.9
6–7	22–91/86	5181	.79	.86	38.94	11.0	53.2

* Personal communication.
† This is now listed as 6 L.35, but spore quartet data place the break in the short arm.

estimated, since pollen sterility was not measured, but presumably these plants had about 50 percent spore abortion.

In interchanges having the break in the long arm of chromosome 6, the frequencies of adjacent-2 segregations are measured by direct counts of the frequencies of 2-diffuse microspore quartets.

In this group of interchanges the highest percentage of the "two diffuse" type was 50.8 as indicated in Table 2. There was a negative correlation between the total lengths of the interstitial segments and the percentage of "two diffuse" type. This is borne out by the following tabulation of average values for the interchanges grouped in 10 μ lengths of their interstitial segments.

Length of intersegment	No. of interchanges	Avg. percent of adjacent-2	Range in percent of adj.-2
0–10.0	5	26.6	11–40.0
10.1–20.0	10	27.1	8–51
20.1–30.0	6	13.7	1–22
30.1–40.0	5	7.7	4–16
40.1–60.0	4	5.9	2–10
>60.1	1	0.0	

The correlation coefficient, $r = -.31$, is very close to that of the five percent level of significance. That means, the longer the interstitial segments, the less adjacent-2 segregation.

The results given in Table 2 show general agreement with those reported by BURNHAM (1950), however, there appear to be a few exceptions having very high values of adjacent-2 segregation, e.g. 3–6(26–70), 6–7(25–49) and 3–6 (x3–3) which have percentages of two diffuse spore quartets of 50.8, 42.5 and 40.0 respectively.

BURNHAM (1948, 1950) found in maize interchange heterozygotes involving chromosome 6 a ratio of 1 alternate:1 adjacent segregations; the latter including the adjacent-1 and adjacent-2 types. In those spore mother cells in which the con-

figuration is a ring and in which there is little or no crossing over in the interstitial segment, this ratio is close to 2:1:1 respectively. In those with long interstitial segments, adjacent-2 segregation is very low in frequency. Spore abortion likewise is close to 50 percent. In the one exception, T8-9a, it was 59 percent.

In the interchanges with the breaks in the satellite, as indicated in Table 3, the percentage of "two diffuse" did not exceed one percent.

Certain plant species show regularly alternate segregation in interchange rings which results in a very low degree of sterility. CLELAND (1936) in his work on

TABLE 2

The ANDERSON *identification numbers, break positions in the chromosomes, total lengths of the interstitial segments and percentages of the "two diffuse" type of spore quartet in plants heterozygous for interchanges with the break in chromosome 6 in the long arm. Interchanges arranged in order of increasing length of interstitial segments*

Interchange	File no.	ANDERSON A.R.S. 34–4 no.	Break in L6	Break in other chromosome	Total length of interstitial segments in μ	Percent of 2-diffuse (Adjacent-2)
6–9	29–39	4505	.13	cent.	4.79	32.6
3–6	X3–3	....	.11	L.02	4.88	40.0
2–6	33–28	5906	.13	S.15	9.22	25.2
6–10e	D–13	....	.14	S.43	9.37	10.9
3–6	32–51	6349	.15	L.10	9.67	24.3
6–7	36–109	6498	.16	S.48	11.86	20.2
6–10d	C–27	....	.16	L.29	13.75	7.9
2–6	27–24	5472,73	.15	L.25	14.76	27.8
3–6	26–70	5368	.20	L.22	16.49	50.8
2–6	34–131	6931	.23	L.24	17.34	24.4
6–7	25–49	4573	.22	L.27	17.37	42.5
4–6	33–82	6623	.31	L.18	17.95	20.7
5–6	37–95	5765	.32	L.19	17.96	16.0
1–6	30–80	5537	.22	S.31	19.22	34.0
6–8	Li	166a	.43	S.36	19.88	26.4
1–6	21–60	5072	.45	S.17	22.67	21.6
4–6b	.....	....	.16	S.80	23.87	16.5
6–8	24–3	6187	.19	L.51	25.47	21.1
5–6a	.....	....	.43	L.35	27.19	18.7
6–7	25–62	4594	.52	S.67	27.48	3.0
6–9	30–102	5622	.42	S.94	29.76	1.4
6–10a	.....	....	.75	L.15	31.69	3.7
5–6	35–72	6062	.78	L.20	35.21	7.2
1–6	25–6	....	.30	L.71	36.52	7.9
6–10	37–106	5980	.93	L.13	37.77	3.5
1–6	70–4	7097	.62	S.46	39.33	16.3
3–6	70–72	7162	.53	L.52	41.08	6.5
5–6	3773–6	5622	.42	S.94	41.19	2.3
5–6	25–78	4666	.86	L.35	43.03	5.3
5–6	3251–2	5964	.89	L.34	43.80	9.4
	31–50		.48	L.83	44.60	9.7
2–6f	84	....	.87	L.79	61.24	0.0

TABLE 3

The file numbers, breaks in the chromosomes, total lengths of the interstitial segments and percentages of the two-diffuse type of spore quartets in the interchanges with the break in the satellite

Interchange	File no.	Anderson A.R.S. 34–4 no.	Break in S6	Break in other chromosome	Total length of interstitial segments in μ	Percent of 2-diffuse (Adjacent-2)
4–6	29–137	5227	SaT.	S.46	22.25	0.99
6–9	37–1	4778	SaT.	L.30	20.32	0.40
6–7	24–65	7036	SaT.	L.63	21.63	0.29

Oenothera has stated that adjacent chromosomes are separated to opposite poles in a very large majority of cells, that is, they follow the alternate type of segregation.

In Datura, Blakeslee, Bergner and Avery (1937) reported rings with sterility, and without sterility, i.e. alternate segregation.

Smith (1939) in working on an unequal interchange in *Triticum monococcum* found that more than 90 percent of the chromosome arrangements were of the zigzag orientation which resulted in a very low percentage of sterility. Rhoades (as described by Swanson 1957) found directed segregation in a 3-chromosome complex in maize.

SUMMARY

Forty lines heterozygous for interchanges between chromosome 6 and the arms of all the other nine chromosomes were used. They were divided into three groups according to the position of the break in chromosome 6: the first group with the break in the short arm (three interchanges); the second with the break in the long arm (34 interchanges); and the third with the break in the satellite (three interchanges).

It has been found that for the first two groups, the longer the interstitial segment, the lower the frequency of adjacent-2 segregation. However there are a few exceptions having very high values of adjacent-2 segregation, e.g. 50.8, 42.5 and 40.0. These exceptions need further study. For the third group there was little or no adjacent-2 segregation either in those with short or long interstitial segments.

ACKNOWLEDGMENTS

The writer is deeply indebted to Dr. C. R. Burnham for suggesting this investigation.

He also wishes to thank Dr. E. G. Anderson and Dr. A. E. Longley for providing him with the material used in the study, and the facilities for the cytological work in their laboratory at the California Institute of Technology.

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GENETIC FINE STRUCTURE OF THE C REGION AND THE LINKAGE MAP OF PHAGE P22[1]

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Received March 20, 1961

ON infection with "temperate" phage, some bacteria may lyse, but some survive and give rise to lysogenic progeny. Lysogenic bacteria possess the ability to produce the infecting phage but are immune to subsequent infection by the phage. Genetic control of the lysogenic condition lies in the prophage which becomes part of the hereditary material of the bacterium (LEDERBERG and LEDERBERG 1953; LENNOX 1955; JACOB 1955). Temperate phages usually produce plaques with turbid centers owing to the growth of lysogenic bacteria. "Virulent" phages produce plaques with clear centers as they usually lyse all infected cells with concomitant liberation of progeny phages.

The wild type of phage P22 of *Salmonella typhimurium* (hereafter designated c^+) is temperate and produces turbid plaques (ZINDER and LEDERBERG 1952). Clear-plaque forming mutants, affected in the ability to become prophage, have been isolated. All such mutants fall into one of three phenotypic groups; c_1, c_2 and c_3 (LEVINE 1957). The mutants of class c_3 are still temperate, but the frequencies of lysogenization with these are lower than with c^+. The clear mutants c_1 and c_2 behave like virulent phages. However, in mixed infection with mutants of the c_2 type, phages of the c_1 type lysogenize as well as the c^+ phage. This interaction between virulent phages in mixed infection resulting in lysogeny has been called co-operation (KAISER 1957). Preliminary genetic studies placed the c mutations in a localized region of the phage P22 linkage map. The present report describes a detailed fine mapping of this region leading to its division into three complex genetic loci corresponding to the three phenotypic groups of mutants.

MATERIALS AND METHODS

Bacteriophage strains: The c^+ type of the *Salmonella typhimurium* phage P22, a large number of independently arising spontaneous c mutants, and some plaque-morphology mutants were used.

A complete description of the characteristics of the three classes of c types and a spot test for their rapid identification has been given (LEVINE 1957). No ex-

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ceptions to this classification have been detected among the more than 150 spontaneous *c* mutants tested. The plaque-morphology mutants, as scored on indicator agar (see below) have the following characteristics:

$+$:	wild type; dark green ring
m_1:	pale green ring with somewhat irregular edge
m_3:	dark green ring with golden brown halo
m_4:	brown-green ring with irregular edge
h_{21}:	narrow pale green ring with smooth edge; called γ by ZINDER
g:	broad dark green ring with smooth edge
m_3h_{21}:	narrow pale green ring with golden brown halo
m_3g:	broad dark green ring with golden brown halo
m_1m_3:	pale green ring with golden brown halo
gh_{21}:	narrow dark green ring with smooth edge
gm_4:	darker green than m_4; somewhat similar to wild type but with a brownish cast and an irregular edge.

h_{21} with m_1 and m_4 cannot be distinguished by inspection.

Bacterial strains: Salmonella typhimurium strain LT2 and its mutants were used as host bacteria in these experiments.

Media: The following media were employed: L broth, indicator agar (BRESCH 1953), soft agar for top layers, EMB galactose agar and buffered saline. The methods of preparation of these media are as previously described (LEVINE 1957).

Experimental procedures

Galactose positive bacteria of strain LT2 are grown to a concentration of about 10^8 cells/ml in L broth. The cells are washed twice in buffered saline, resuspended to one half the original volume and aerated for 45 minutes at 37°. The cell concentration is then readjusted to 10^8 cells/ml with buffered saline. These bacteria are infected with equal multiplicities, ten particles, of each of the two parent phage stocks. Adsorption is at least 95 percent completed in five minutes. A 1:10 dilution is made in L broth containing antiphage serum in concentration sufficient to inactivate 99 percent of the unadsorbed phage in five minutes. Further dilutions are made and 0.05 ml samples are plated on EMB galactose agar with a heavy inoculum of a *Gal*$^-$ phage sensitive mutant. Plating on EMB galactose agar permits the detection of infective centers and surviving bacteria which may or may not be lysogenic (LEVINE 1957). After 90 minutes, when the rise period is completed, chloroform is added to the dilute lysate to kill any surviving bacteria. Diluted samples are plated on indicator agar for examination of the phage liberated by lysis. Mixtures of parental phages are irradiated in buffered saline before infection with ultraviolet light in some of the crosses to be reported below. The ultraviolet source used was a 15 watt G.E. germicidal lamp at a distance of 50 cm for exposures of 105 seconds. Approximately five percent of the exposed particles survive this dose.

RESULTS

Crosses were undertaken between a large number of *c* mutants to study the genetic structure of the *c* region. The expected recombinants between two non-identical *c* mutants are the double mutant and its reciprocal, the turbid c^+. The double mutants are not easily distinguished among the progeny and are therefore not of much use in such studies. On the other hand, the turbid plaques of the c^+ recombinants are easily detected and the frequency of these among the total progeny of a mixed infection can be determined with accuracy. In addition, this determination is not complicated by back mutation as no case of reversion from *c* to c^+ is known in this material. Some 300,000 progeny from control infections with all of the individual mutants used in these experiments have been carefully screened without observing any c^+ phages. Many of these were exposed to ultraviolet light before infection and still no reverse mutations were found. Many tens of thousands more have been examined less carefully with the same result. It seems quite clear that these c^+ phages arise by recombination as they are found only among the yields of mixed infection with independently isolated *c* mutants. The frequencies of c^+ recombinants from these mixed infections are, then, taken as the measure of linkage between parental *c* mutants and these frequencies are doubled (on the assumption that the reciprocal double mutants are formed as frequently) in the data to be presented.

The c^+ recombination data (Table 1) from two factor crosses with 16 *c* mutants support the previous conclusion (LEVINE 1957) that the *c* mutants fall into a circumscribed region of the phage chromosome. The distribution of individual mutations within this region is, however, not random. Rather, there is a clustering of mutations of similar phenotype, dividing the region into three parts. Into one genetic group fall all the mutations previously classified as type c_1. These are closely linked with the region into which all the c_2 type mutations fall. These in turn are linked to the region in which the three c_3 mutations lie. Within each of these groups, the individual mutations map as specific sites which can be arranged in a linear order. The *c* region, then, is made up of three closely linked complex genetic loci, c_1–c_2–c_3, corresponding to the three phenotypic classes of *c* mutants. Each locus consists of multiple sites at which mutations may occur, affecting that particular phenotype, and these sites may recombine with one another. These facts are graphically illustrated by the linkage map of the *c* region in Figure 1.

To extend the fine mapping of the *c* region, use was made of the finding (JACOB and WOLLMAN 1955) that ultraviolet irradiation of parental phages before infection results in increased frequencies of phage recombination. The dose employed gives maximum recombination values in phage P22. The UV linkage map of the *c* region of phage P22 is shown in Figure 2. These results extend those given above and are in good agreement with them. Again there are no exceptions to the grouping of mutations according to phenotype into the three distinct loci. Also there is surprisingly good correspondence between the two maps in the order of the same mutational sites within these loci.

TABLE 1

Genetic recombination in the lytic yield of two factor crosses between c *mutants*

Parents†	Total c^+ recombinants	Total progeny	Percent recombination (×2)
Intergroup crosses			
$c_1^{7} \times c_2^{5}$*	87	17,157	1.014
$c_1^{65} \times c_2^{5}$	37	15,377	0.481
$c_1^{49} \times c_2^{5}$*	214	50,747	0.843
$c_1^{65} \times c_2^{27}$	0	4,341	0.00
$c_1^{52} \times c_2^{27}$	24	11,125	0.432
$c_2^{5} \times c_3^{77}$*	103	25,381	0.812
$c_2^{5} \times c_3^{11}$*	41	8,099	1.01
$c_2^{5} \times c_3^{41}$*	85	7,499	2.27
$c_1^{65} \times c_3^{77}$	71	10,338	1.37
Intragroup crosses			
$c_1^{65} \times c_1^{60}$*	79	127,997	0.123
$c_1^{65} \times c_1^{49}$*	121	162,678	0.149
$c_1^{65} \times c_1^{7}$	96	126,627	0.152
$c_1^{65} \times c_1^{45}$*	111	111,873	0.198
$c_1^{65} \times c_1^{52}$*	106	96,632	0.219
$c_1^{60} \times c_1^{49}$	0	60,871	0.000
$c_1^{60} \times c_1^{7}$	0	97,807	0.000
$c_1^{60} \times c_1^{31}$*	17	94,216	0.0361
$c_1^{60} \times c_1^{52}$*	12	63,628	0.0377
$c_1^{60} \times c_1^{3}$*	37	110,861	0.0668
$c_1^{49} \times c_1^{52}$	38	122,656	0.0620
$c_1^{7} \times c_1^{52}$	15	122,970	0.0244
$c_1^{45} \times c_1^{52}$	1	113,596	0.0018
$c_1^{31} \times c_1^{3}$*	17	155,853	0.0218
$c_1^{52} \times c_1^{3}$	2	109,357	0.0037
$c_2^{5} \times c_2^{8}$*	153	116,474	0.263
$c_2^{5} \times c_2^{23}$*	143	103,756	0.276
$c_2^{5} \times c_2^{27}$*	463	185,659	0.499
$c_2^{8} \times c_2^{23}$*	15	125,848	0.0238
$c_2^{8} \times c_2^{27}$*	162	79,420	0.408
$c_2^{23} \times c_2^{27}$*	284	170,537	0.333
$c_3^{41} \times c_3^{77}$	0	4,300	0.00
$c_3^{41} \times c_3^{11}$	0	4,877	0.00
$c_3^{11} \times c_3^{77}$	0	5,892	0.00

† Parent phages were used at m.o.i. of ten each.
‡ Two other loci, m_3 and h_{z_1}, were followed in these crosses (see text).

Some *c* mutations behave anomalously in that they recombine with certain alleles to produce distinctive frequencies of c^+ progeny, but recombine with lower than expected frequencies or not at all with others. These comments are particularly pertinent to the c_3 locus. No c^+ recombinants are observed in the admittedly small samples of progeny from crosses between c_3^{11}, c_3^{77} and c_3^{41} (Table

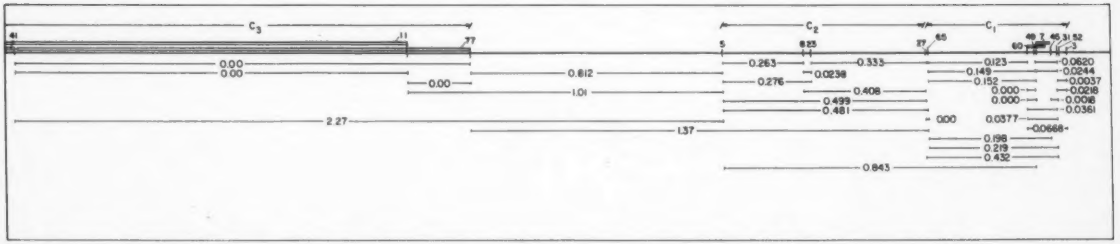

FIGURE 1.—Linkage map of *c* mutations. The order and distances are based on the uncorrected frequencies of recombination for the two factor crosses listed in Table 1. The open bars above the map indicate alleles which may be multisite mutations (see text).

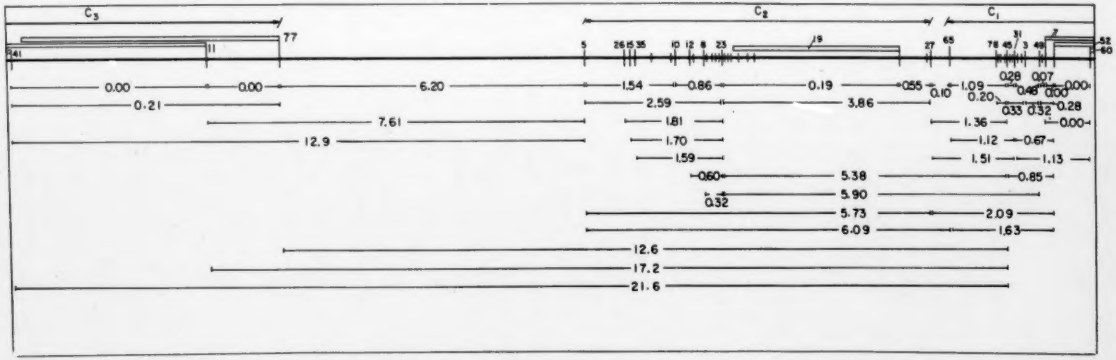

FIGURE 2.—The ultraviolet linkage map of *c* mutations. The map is based on recombination frequencies for two factor crosses in which mixtures of parental *c* phages are irradiated with ultraviolet light before infection. The position of the numbered alleles can be assigned with some assurance. The positions of unnumbered alleles (indicated on the map by the shorter perpendicular lines) are only approximate. The open bars above the map indicate alleles which may be multisite mutations (see text).

1 and Figure 1). Following ultraviolet treatment, only 0.21 percent recombination is found in the $c_3^{77} \times c_3^{41}$ cross and no recombinants are observed in the other two crosses (Figure 2). These findings suggest that the c_3 locus is very small indeed. However, in crosses to mutant c_2^5 all three c_3 mutants give quite high frequencies of recombination: 0.812, 1.01 and 2.27 between c_2^5 and c_3^{77}, c_3^{11} and c_3^{41} respectively (Table 1 and Figure 1). These data suggest that the c_3 locus is rather long and that the individual c_3 mutations cover extensive and common segments. The possibility that the c_3 mutations (and a few others) are multisite mutations (DEMEREC and HARTMAN 1959) is indicated by the open bars above the linkage maps in Figures 1 and 2. These conflicting considerations make difficult the determination of the precise dimensions of the c_3 locus.

A number of four factor crosses have been carried out involving, in addition to the two c alleles, the m_3 and h_{21} plaque morphology markers. The order of the c mutations relative to one another and to the plaque morphology markers can be determined from the distribution of these markers and their wild-type alleles among the c^+ recombinant progeny. These crosses are designated by the asterisks in Table 1. Such analyses confirm the positions of the c mutations (as shown in Figure 1) wherever applicable and also establish the order m_3–c_3–c_2–c_1–h_{21}.

A complete linkage map (Figure 3) for phage P22 has been drawn combining the data from Table 1 with the recombination data from the crosses listed in Table 2. The recombination frequency between the h_{21} and m_4 loci has not been determined directly. The map distance given in Figure 3 is intended only as a rough approximation, derived by the difference between the distance from g to h_{21} and that from g to m_4.

DISCUSSION

All clear-plaque forming mutants of phage P22 can be classified into one of three groups based on the ability to lysogenize. The genetic evidence demon-

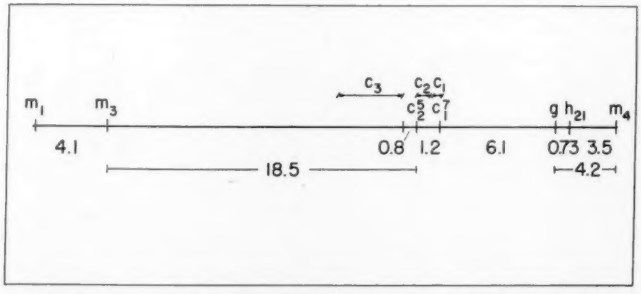

FIGURE 3.—Linkage map for phage P22 mutations. The order and distances between plaque-morphology markers and plaque-morphology markers and c mutations are based on the crosses listed in Table 2 and on the four factor crosses mentioned in the text. The distance between h_{21} and m_4 is only an approximation, derived from the difference between the map distance from g to h_{21} and that from g to m_4. The distances between c mutations are derived from Figure 1.

TABLE 2

Genetic recombination in the lytic yield of crosses involving plaque morphology mutants

Parents*	Percent recombination										Total progeny
	m_1–m_2	m_2–c_2^s	m_1–c_2^s	c_2^s–h_{21}	m_2–h_{21}	c_1^7–h_{21}	c_1^7–g	g–h_{21}	c_1^7–m_4	g–m_4	
$m_1 + c_2^s \times + m_2\, c^+$	4.1	18.8	21.5	...	...	...	...	...	...	...	994
$m_2\, c^+\, h_{21} \times + c_2^s +$	...	18.3	...	7.9	22.1	...	...	...	...	...	1801
$c_1^7\, h_{21} \times c^+ +$	...	...	...	...	...	7.0	...	...	...	...	1683
$c^+\, g + \times c_1^7 + h_{21}$	...	...	...	...	...	6.4	5.9	0.75	...	...	3893
$g\, h_{21} \times + +$	...	...	...	...	...	...	...	0.72	...	...	4566
$c^+\, g\, m_4 \times c_1^7 + +$	...	...	...	...	...	...	6.6	...	8.1	3.9	1228
$c_1^7\, g\, m_4 \times c^+ + +$	...	...	...	...	...	...	6.4	...	7.9	4.5	1605

* Parent phages were used at m.o.i. of ten each. The parental factors are given in the order in which they are linked.

strates that the corresponding mutations occur at three closely linked but non-overlapping complex loci or pseudoallelic series which behave like the functional units or cistrons of BENZER (1957). The mutations of each series are separable by recombination into different sites. The alleles of the different loci interact physiologically (complementation) to give lysogeny, whereas the alleles of the same locus do not.

A remarkably similar system of co-operation between clear mutants leading to lysogeny has been reported in coliphage λ (KAISER 1957). Three different phenotypes of clear mutants have been recognized, which fall into three adjacent regions. Mixed infection with a pair of phenotypically different mutants, each of which lysogenizes very poorly or not at all, produces the high frequency of lysogeny typical of the wild type. Clear-plaque forming mutants that show co-operation are also found in phage P2 (BERTANI 1960) and in phage D3 of *Pseudomonas aeruginosa* (EGAN and HOLLOWAY 1961).

It has been proposed (LEVINE 1957) that at least two steps are required for the establishment of lysogeny by phage P22. Phage c^+ can carry out both steps; c_1 mutants are blocked in step 1, c_2 mutants fail in step 2. The phenotypically different mutants each provide, in mixed infection, the function missing in the other, resulting in physiological complementation leading to lysogeny. The c_1 functional unit of phage λ, analogous to the c_2 of P22, determines the locus occupied by the prophage on the chromosome of *E. coli*, the immunity specificity of the phage (KAISER and JACOB 1957) and also controls the production of an immunity substance (JACOB and CAMPBELL 1959). Immunity specificity in phage P22 does not appear to be controlled by the c region. ZINDER (1958) has evidence for an immunity locus near the m_3 end of the phage P22 linkage map. The c_2 cistron of this phage may retain some aspect of control of integration of prophage into the bacterial chromosome. This is suggested by the fact that P22 phages carrying c_2 mutations never become prophages (LEVINE 1957). Finally, there are suggestions that the functions carried out by the different functional units may take place at different times after infection (KAISER 1957; TING 1960).

Clear plaque mutants appear among the phages of Serratia (KAPLAN, WINKLER and WOLF-ELLMAUER 1960). In contrast to the phages P22, λ, P2 and D3, no co-operation leading to lysogeny was found in tests among seven X-ray and five ultraviolet-induced c mutants of Serratia phage χ. These mutants also show reversions to temperate wild type. Further analysis of the control of temperateness in this phage is clearly indicated.

SUMMARY

All clear-plaque forming mutants of phage P22 can be classified into three groups; c_1, c_2 and c_3. Genetic fine mapping studies show that the corresponding mutations occur at three adjacent complex loci in which the individual mutations are linearly arranged. A complete linkage map for phage P22 is given.

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THE PROCESSES OF SPONTANEOUS RECOMBINATION IN VEGETATIVE NUCLEI OF ASPERGILLUS NIDULANS[1,2]

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Received July 14, 1961

IN the normally haploid strains of the fungus *Aspergillus nidulans* stable diploid strains are produced spontaneously as a rare event by fusion of nuclei (ROPER 1952). Such spontaneous diploids can easily be isolated from heterokaryons between differently marked haploid strains. They are visually distinguishable from the haploid since they show less regularly arranged and less uniformly shaped conidial heads. The size of the individual conidia increases proportionally with ploidy and provides an easily measurable criterion of the number of chromosome sets. That the diploid state is abnormal in some respects is indicated by the fact that diploid strains are almost completely sterile, meiosis of the diploid proceeding only up to metaphase I (ELLIOTT 1960). Also, in competition, the haploid shows the higher viability. Diploids are fairly stable, but produce rare mitotic recombinants, some of which are again haploid (ROPER 1952; PONTECORVO and ROPER 1953). So far, little is known about the occurrence of diploidy in nature and therefore about the actual evolutionary contribution of this potentially efficient type of parasexual recombination (PONTECORVO 1956). Also, while some mitotic segregation has been found in a variety of organisms, the question of its general occurrence in fungi and in tissues of higher organisms is still open.

Ever since the discovery of mitotic recombination in diploid strains of *Aspergillus nidulans*, attempts have been made to analyse the underlying processes. While one process, mitotic crossing over, was recognized early (PONTECORVO, TARR-GLOOR and FORBES 1954), and has been analysed in detail (PONTECORVO and KÄFER 1958), it was more difficult to elucidate the mechanism(s) producing the other types of segregants (haploids, nondisjunctional diploids and aneuploids). It became clear that for the analysis of these mitotic recombinants, which are all products of some kind of chromosomal segregation, it was necessary to use diploids with markers on all chromosomes, preferably on both chromosome arms. Mapping procedures making use of mitotic recombination have therefore been worked out, the available useful markers mapped and crossed together in suitable arrangements and the chromosome number established genetically (KÄFER 1958). Various chromosomal aberrations, especially translocations, were encountered and it became possible to recognize several types of characteristic distortions of the patterns of mitotic recombination caused by their presence in heterozygous condition.

[1] This work has been supported by a grant from the National Research Council of Canada.
[2] Cost of extra pages in this article will be paid by the Author.

The present work was undertaken to elucidate the process or processes of mitotic *chromosomal* segregation, making use of well-marked diploids and non-selective methods for the isolation of segregants. The aim was to gain a detailed knowledge not only of the basic process(es) but of the many other factors which obviously influence the frequency of the recovered recombinants (e.g. presence of aberrations, conditions of growth, methods of isolation).

It was hoped that such information could provide decisive criteria for the interpretation of results obtained in the analysis of recombination processes in many asexual species. In some cases a remarkable similarity in the results justifies the assumption that the underlying processes in other fungi are the same as in *Aspergillus nidulans* (e.g. in *Aspergillus niger*, HUTCHINSON 1958 and LHOAS 1961; in *Penicillium chrysogenum*, SERMONTI 1957; in yeast, ROMAN and JACOB 1958; and possibly in *Fusarium oxysporum*, BUXTON 1956). On the other hand, results not following the same pattern are obtained in many cases, either with different strains of the same species (e.g. in Penicillium, especially after treatment with mutagens, MUCHNIK 1961; and in Fusarium, TUVESON and GARBER 1959) or with other species (in Streptomyces, e.g. BRAENDLE and SZYBALSKI 1959).

A better understanding of the processes of mitotic recombination in fungi might also permit a more fruitful comparison with the phenomena encountered in tissues of higher organisms, where it is not possible to breed from recombinant cells. Here analysis must be indirect and evidence is usually based on patterns produced by the phenotypically expressed recombination of genetic markers or by cytologically recognizable variations of the karyotype (e.g. in endosperm of maize, JONES 1937; MCCLINTOCK 1956; or in root tips of Allium, HUSKINS 1948; in body surface cells of Drosophila, STERN 1936; or of Ephestia, KÜHN 1960; or in human cells grown in tissue culture, PUCK 1958).

MATERIALS AND METHODS

Strains: All mutant markers used in this analysis have been induced or selected in the same wild-type strain of *Aspergillus nidulans* which normally possesses a haploid set of eight chromosomes. Details are given elsewhere (KÄFER 1958), except for a new spontaneous mutant, "chartreuse" (*cha*), which determines light green conidial color and is located distal to *ribo2* in the right arm of linkage group VIII (Figure 1). Four suitably marked haploid strains were obtained by intercrossing the original mutant strains isolated after X-ray or ultraviolet irradiation.

To make sure that a standard set of chromosomes would be present for analysis, several strains were checked for translocations (by mitotic analysis) and mutants which could not be separated from aberrations were excluded. In spite of this, it turned out that one of the final strains (diploid R, homologue a, Figure 1), which was expected to have standard chromosomes, carried a translocation between linkage groups VI and VII. It was decided to use this strain in any case, since valuable information on the influence of translocations on mitotic segregation

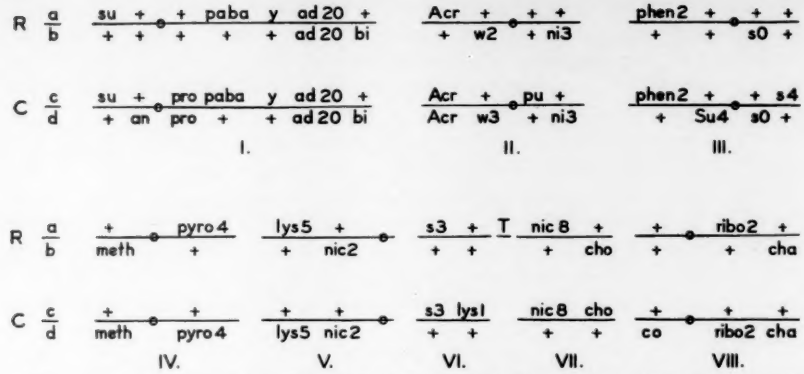

R a/b su + + paba y ad20 + / + + + + + ad20 bi
Acr + + + / + w2 + ni3
phen2 + + + / + + s0 +
C c/d su + pro paba y ad20 + / + an pro + + ad20 bi
Acr + pu + / Acr w3 + ni3
phen2 + + s4 / + Su4 s0 +
I.
II.
III.
R a/b + pyro4 / meth +
lys5 + / + nic2
s3 + T nic8 + / + + + cho
+ ribo2 + / + + cha
C c/d + + / meth pyro4
+ + / lys5 nic2
s3 lys1 / + +
nic8 cho / + +
+ + + / co ribo2 cha
IV.
V.
VI.
VII.
VIII.

FIGURE 1.—Genotypes of repulsion diploid R (synthesized from the two haploids a and b) and coupling diploid C (c/d) with markers on linkage groups I–VIII. Conidial color mutants: *y* = yellow, *w2*, *w3* = white (allelic), *cha* = chartreuse. Colonial mutant: *co* = compact. Mutants determining requirements: *ad20* (allele of *ad8*) = adenine, *an* = aneurin (= thiamin), *bi* = biotin, *cho* = choline, *lys1* and *lys5* = lysine, *meth* = methionine, *ni3* = nitrite, *nic2* = nicotinic acid, *nic8* = nicotinic acid or tryptophan, *paba* = p-aminobenzoic acid, *pro* = proline, *pu* = putrescine, *pyro4* = pyridoxine, *ribo2* = riboflavin, *s0*(*s12*), *s3* and *s4* (allele of *s1*) = sulphite. Suppressors: *su1ad20* (*su*): suppressing *ad20*, *Su4pro* (*Su4*) suppressing *pro*. Resistance mutant: *Acr* = acriflavine. T = translocation (VI–VII). (All unnumbered alleles have isolation number 1).

could be obtained in this way. At the moment it is assumed, but not certain, that the so-called "standard" arrangement of chromosomes present in the other three strains used in this analysis is the same as that of the original wild type.

The two test diploids were selected from heterokaryons between two each of the four well-marked haploid strains, using ROPER's technique (1952). One of these diploids showed an extremely early mitotic crossing over in linkage group VIII before analysis was started. The new diploid (diploid C, Figure 1) carried the two reciprocal crossover strands, with *cha* and *ribo2* heterozygous but now in coupling, while each haploid strain had carried one of the markers. The two diploids used for analysis carried markers on all eight linkage groups. Their complete genotype is given in Figure 1. In the repulsion diploid R it was possible to follow segregation of all chromosomes (because of the presence of the translocation VI–VII the only unmarked chromosome of linkage group VI is not found to segregate independently). The coupling diploid C has 23 heterozygous markers, wherever possible arranged in coupling on both chromosome arms to permit distinction of nondisjunctional types from mitotic crossovers showing segregation in single chromosome arms; but it has the disadvantage that in five linkage groups (IV–VIII) one homologue does not carry any markers. The most likely positions of six of the centromeres are indicated in Figure 1 (full evidence only available for linkage groups I and II, PONTECORVO and KÄFER 1958).

Techniques: Standard media (KÄFER 1958) and testing methods (PONTECORVO, ROPER, HEMMONS, MACDONALD and BUFTON 1953) were used. To obtain

and analyse mitotic segregants, platings of samples of conidia on complete medium (CM) were made. A new plating method, "needle plating" was used extensively to plate samples from very small or badly conidiating colonies (or parts of colonies): conidia were collected from one or several conidial heads by touching with a fine needle and rinsing off in 0.2–1.5 ml of saline. Platings onto CM plates were made from these suspensions without diluting or counting and usually showed the desired low density of a few colonies per plate.

Allelism tests were made for the various sulphite-requiring mutants which can easily be characterized by their ability to crossfeed alleles of different genes, but not of the same gene, on medium without sulphite. Complementarity tests needed to determine the presence of other epistatic markers in haploids have not been carried out, since no case occurred where crucial evidence could have been obtained in this way.

EXPERIMENTS AND RESULTS

The experiments consisted simply of conidial platings from two well marked diploids under optimal conditions and detailed analysis of all segregants obtained in this way. The detailed procedure was as follows: A small number of colonies ("primary colonies") was grown from single uninucleate conidia from each of the two diploids and the genotype of these nuclei was verified by isolating mitotic haploids from the conidia in the center of each of these colonies. To analyse the mitotic segregation in these primary colonies batches of conidia (about 10^6) were harvested from each of them, diluted, and a small sample of conidia (usually several hundred, for details see Table 1) plated at low density on complete medium (CM). Most of these conidia are of the original type, while the rare segregant nuclei, produced by mitotic recombination during the growth of the primary colonies, are present only in very few of the conidia. The recombinant conidia form colonies of "aberrant" types which, on objective criteria, fall into two classes (1) colonies with mutant phenotype and normal growth (stable haploid and diploid segregants) and (2) colonies with abnormal growth patterns (e.g. unstable aneuploids are expected in this class).

1. Of the *stable* segregants which grow normally, but show a mutant phenotype, one category can be detected visually by inspecting all colonies grown from the plated conidia, namely (a) those showing mutants which affect the conidial *color* (*y*, *w*, *cha*); the others, (b) showing nutritional *requirements*, can only be found by transfer to minimal medium (MM). Only part of the colonies from each plating were tested on MM for requirement (Table 1). To characterize the "color" segregants and the "requiring" segregants, their phenotypes and ploidy are determined by further tests.

2. The *unstable* segregants producing abnormal growth patterns are also detected visually. All colonies deviating in size or conidiation are designated as "*abnormal*" segregants. They are further analysed by replating conidia from the center of each one and testing the obtained colonies as well as the stable sectors many of them produce.

TABLE 1

Size of analyzed samples and frequency of segregants

	Numbers		Frequency	
	Diploid R	Diploid C	R	C
Inspected colonies				
Number of platings	19	13	..	..
Colonies per plating (range)	169–1189	61–687	..	..
Total colonies	9877	3595	..	..
Average density: colonies/plate	20	10	..	..
Colonies tested for requirements				
From original platings	2054	1893	..	..
Secondary isolates	3520	1446	..	..
Total	5574	3339	..	..
1. Stable segregants				
(a) "color" segregants (3 markers)				
Whole colonies	19	16	0.2%	0.4%
Mixed colonies	6	1	..	..
(b) "requiring" segregants (14 chromosome arms marked)				
From original platings	12	7	0.7%	0.5%
From secondary isolates	27	10		
2. Unstable segregants				
"Abnormals", mostly aneuploids sectoring,				
With segregant sectors	41	36	0.6%	1.3%
With sectors like centers	14	12		
"Lost", probably sectoring,				
Some sectors recovered	14	11	0.3%	0.4%
No segregant recovered	16	3		
Translocation segregants				
With segregant sectors	22	..	0.2%	..
With sectors like centers	1?	..	..	..
"Misjudged", later normal				
Analysed	17	6	..	..
Not analysed	11	2	..	..

This method of isolation of segregants is completely nonselective and suitable for recovery of types with reduced viability which have been missed in earlier experiments. It has the disadvantage that each batch of conidia, harvested from the surface of a "primary" colony, contains segregant clones of various sizes, depending on the size of segregant patch formed from a single event in the primary colony. The experiments were therefore originally designed to permit calculation of the absolute frequency of each type of segregant with statistical correction for clonal distribution (LURIA and DELBRÜCK 1943). However, the size of the different sample platings varied considerably because the density (average given in Table 1) was decreased during the course of the experiments (since it turned out that only low densities of less than ten colonies per plate permit recovery of practically all the very slow growing types). Also the size of each sample was found to be small enough and the segregation events rare enough that

even relatively frequent types occurred in not more than about one quarter of the independent platings. It seemed therefore to be a reasonable assumption that the few cases of two or three segregants of exactly the same type within one sample all represent clones, each from a single event. They are treated as such throughout the analysis and a minimum estimate of frequencies is calculated: number of independent events per total number of tested colonies (Tables 1, 2 and 6).

Isolation of stable segregants: (a) Most "*color*" *segregants*, detected visually in the original platings, were isolated as colonies with pure mutant color and normal growth pattern. In a few cases, however, mixtures of mutant and wild type color were analysed (Table 1). It was hoped that in some of these cases, where segregation occurred in one of the first mitotic divisions, a "twin spot" consisting of the two complementary, phenotypically recognizable, products of mitotic crossing over might be recovered. One of the five cases analysed from diploid R was such a "twin spot" (the two diploid recombinant types were *paba y bi*$^+$ and *paba*$^+$ *y*$^+$ *bi*, resulting from a mitotic crossing over in linkage group I between *paba* and the centromere). Only one further such twin spot was encountered: two reciprocal diploid sectors from diploid R (homozygous *cha* and *ribo2* respectively, resulting from a single event of mitotic crossing over in linkage group VIII) were present among the sectors of a single unstable colony. Two other cases, showing high frequency of apparent twin spotting, will be discussed later.

(b) Under "requiring" segregants are included all those which differed from the original diploids not only with respect to requirement but also (from diploid R) with respect to resistance to acriflavine. They are detected by testing on MM, on which the original diploids (*ad20/ad20 su1ad20/*+) show adaptive growth and form sectors (*su1ad20/su1ad20*) by mitotic crossing over, so that an additional requirement of homozygosis for *su1ad20* or *su1ad20*+ can be detected (similarly on CM supplemented with acriflavine homozygosis for *Acr* or *Acr*$^+$ can be detected; PONTECORVO and KÄFER 1958). Two sets of such segregants were obtained, one consisting of the requirers found among the normal looking colonies of the sample platings which were transferred to MM for this purpose, and a second one resulting from incidental events of segregation in replatings of "abnormal" colonies (secondary isolates), which produced rare requiring sectors (Table 1).

Testing and classification of stable segregants: The phenotypes of the stable segregants were determined by replication on the various differently supplemented test media. The ploidy of these segregants was judged by several criteria; haploids can be distinguished from diploids visually (they show very dense and regular conidiation), by the absence of heterozygosity (testable markers: *su1ad20*, *Acr*, *y*, *w* and *cha*) and by the presence of all the mutants carried on one homologue of each linkage group. Only two were found (among the yellow segregants, Table 2). All other segregants, classified as diploids, were found heterozygous for at least two markers, and generally showed only segregation for mutants of a single chromosome. Three color segregants were exceptions (two of

them homozygous *su1ad20*, and one, from diploid R, *pyro4*) and probably were produced by two consecutive events of segregation. In these and in a few other cases the diameter of the conidia was measured which provides a reliable criterion for distinction of the various levels of ploidy in stable (euploid) segregants (ROPER 1952).

Further classification of the diploid segregants into mitotic "crossovers" or "nondisjunctionals" was possible in all those cases, where segregation involved a chromosome with markers located on both chromosome arms: They are likely to be products of mitotic crossing over when only markers of one arm are found homogygous, and "nondisjunctionals" when all markers on both arms have become homozygous simultaneously (coincidence of crossing over in both arms being very rare, PONTECORVO and KÄFER 1958). Most color segregants (except the *cha/cha* types from diploid R) are classifiable in this way and their frequencies are given in Table 2. The number of "requiring" segregants classifiable for the two types is so small (24) that no comparable frequencies are calculated. Of the 11 from diploid R and 13 from diploid C (listed among the "requiring" segregants of Table 1), 18 were mitotic crossovers, six nondisjunctionals.

Isolation and testing of "abnormal" segregants: "Abnormal" segregants were detected in the original platings as colonies which showed abnormal growth rate and/or conidiation after two days at 35°C. Since all earlier identified aneuploids showed abnormal growth, this class was expected to include aneuploid types which could provide information about the process of chromosomal segregation. To avoid any bias, it was decided to classify as "abnormal" any colony deviating

TABLE 2

Frequency of the different types of stable "color" segregants

Color and phenotype			Number: Diploid R	Diploid C	Total		Frequency
Yellow	*y*	Mitotic CO	0	2	2		
(I R)	*paba y*		5	2	7	9	0.07%
	su paba y	Nondisj.	1	3	.	4	0.03%
		Haploids	2	0	.	2	0.015%
White							
(II L)	*w*	Mitotic CO	5	2	.	7	0.05%
	w ni3	Nondisj.	0	1	.	1	0.007%
		Haploids	0	0	.	0	<0.01%
Chartreuse	*cha*	Mitotic CO	(6*)	2	2		
(VIII R)	*cha ribo2*	(in C)	.	3	3	5	0.14%
	cha ribo2 co	Nondisj.	.	1	.	1	0.03%
		Haploids	0	0	.	0	<0.01%
Average mitotic crossing over/chromosome arm							2 × 0.07%
Average nondisjunction/chromosome							2 × 0.02%
Total haploids						2	0.015%

* Mitotic crossovers not distinguishable from nondisjunctionals.

at all from the majority of normally growing ones. In several cases colonies showing slight variations in appearance were classified as segregants, but later turned out to be normal by all criteria; these cases are presumed to be caused by slight environmental differences and are listed in Table 1 as "misjudged." To analyse all other, genetically "abnormal" segregants, conidia from their centers were harvested and plated on CM. In most cases colonies grown from these conidia showed a deviant phenotype like the original segregant, but generally grew larger and regularly produced better growing sectors under the favorable conditions of a low density plating with almost no competition. It was found that simple transfer of conidia from the unstable centers of tiny "abnormal" colonies only led to the recovery of one or a few stable second order segregants, since the unstable center type was not able to compete. In a few cases, the center type was therefore not recovered, while several sector types were obtained; other tiny colonies were too close to normal colonies and could not be analysed at all (both these groups are listed as "lost" in Table 1). The method of needle plating used in the later part of the analysis permits recovery of most very abnormal types, provided they form a few conidial heads. When the sectoring or otherwise abnormal types were very rare in the first replating of a segregant a secondary replating was made using the conidia from the center of one of them. However, only segregants analysable in the first replating are sure to have been present in the analysed sample; later ones may possibly represent new events of segregation. This slightly reduces the accuracy of the frequencies calculated for the various "abnormal" segregant types. All colonies originally designated as "abnormal" are listed in Table 1, classified by objective criteria, except for segregants interpreted as caused by the presence of the translocation in diploid R. All "abnormals" which could be replated were analysed by testing and classifying at least 26 isolates, mostly stable sectors, from each plating (using the same procedure as for stable segregants). The total numbers of all these tested sectors are listed as "secondary isolates" in Table 1.

Classification of "abnormal" unstable segregants

The "abnormal" segregants turned out to be of a variety of types. Their appearance on the original plating as well as their recovery in secondary replatings depends to a large extent on the plating conditions (especially crowding) and on the number of mitoses preceding the occurrence of secondary segregation events. Meaningful classification of the original appearance (attempted with a similar group of abnormals from an earlier diploid, KÄFER 1960) was therefore not possible. But it has been found that abnormal segregants replated under standard conditions at very low density show a growth pattern characteristic for their genetic constitution. Most of them show a slowly growing central part of characteristic size and reduced conidiation of a specific type (except for tufts of conidiating aerial mycelium in the very center), and form at the edge better growing sectors with normal, diploid or haploid, growth characteristics (see Figures 2–5). They were objectively classified into 11 different types according to the mutants expressed by the unstable type of the center and the pattern of segrega-

tion shown by the sectors. The 11 types are interpreted to represent six major groups (a–f) as follows:

(a) *Hyperdiploids:*

(1) centers are heterozygous (like the original diploid), producing two types of sectors, some like the center and some of a mutant type, nondisjunctional for a single chromosome;

(2) centers are mutant, nondisjunctional for one chromosome, giving two types of sectors, some like the centers and others nondisjunctional for a second chromosome as well;

(3) centers are heterozygous (like the original), producing four types of sectors which show nondisjunctional segregation for the markers of two chromosomes;

(4) centers are of heterozygous (original) or mutant (nondisjunctional) types, the sectors showing nondisjunction for three or four chromosomes in various combinations.

(b) *Unstable types, sectoring without segregation of markers (unmarked chromosomes, diploid C):*

(5) heterozygous centers giving sectors of the same type (all like the original diploid);

(6) centers and sectors are of the same mutant phenotype (nondisjunctional for a single chromosome).

(c) *Hyperhaploids:*

(7) centers are mutant producing haploid sectors only.

(d) *Breakdown types:*

(8) centers produce a variety of nondisjunctional or haploid sectors.

(e) *Lost unstable types:*

(9) centers lost, but stable segregants recovered;

(10) no segregant recovered.

(f) *Translocation segregants:*

(11) (from diploid R only) centers show sectoring; any diploid sectors only segregate for markers of linkage groups VI or VII, while haploid sectors only segregate for all other markers.

(a) *Hyperdiploids:* The majority of segregants were of type (1), that is their centers were heterozygous for all mutant markers, like the original, and produced diploid sectors of two types, one like the original diploid, the other nondisjunctional for a single chromosome. Segregants of type (2) showed the same type of sectoring with segregation for a single chromosome but were in addition homozygous for markers of another chromosome. In 24 cases the segregating chromosome carried markers on both arms and all sectors obtained were recognizably of a nondisjunctional type, segregating simultaneously for the markers on both arms. The ratio of heterozygous:homozygous sectors was roughly 2:1, somewhat lower if the homozygous sectors showed mutant color and were preferentially isolated for testing, somewhat higher if the homozygous mutant sectors had slightly reduced viability (details for all these segregants are shown in Table 3).

TABLE 3

Hyperdiploid segregants showing high frequency sectoring for one linkage group

Inferred type of original aneuploid	Linkage group	Diploid R				Diploid C			
		Homologue				Homologue			
		Segregating	Homozygous	No.	Sector ratio +:nondisj.	Segregating	Homozygous	No.	Sector ratio +:nondisj.
2n + 1	I	Ia	. .	1	11:10	Ic	. .	1	9:10
		Ib	. .	4	97:23				
2n + 2						Ic	IVd	1	10: 5
						Ic	Vd	1	15: 5
2n + 1	II	IIa	. .	1	26:13				
		IIa-b*	. .	1	3 types				
		IIb	. .	2	22:26	IId	. .	1	17: 8
2n + 2		IIa	VIIIa	1	5: 6	IIc	IVd	1	30: 6
2n + 1	III	IIIa	. .	5	87:38	IIIc	. .	2	34:11
		IIIa-b*	. .	1	3 types				
		IIIb	. .	5	147:66	IIId	. .	2	24: 8
2n + 2		IIIa	Vb	1	15: 2	IIIc	Vc	1	15:11
		IIIb	Ia	1	22: 4				
		IIIb	IVa	1	26: 5				
2n + 1	IV	IVb	. .	1	3: 2				
2n + 2						IVd & IVc-d	VIIc	1	45:10
2n + 1	V	Vb	. .	2	30:13				
2n + 1	VI	. .				VIc	. .	3	47:21
2n + 2						VIc	Vc	1	15: 6
2n + 2	VII	VIIb	IVa	1	10:10				
2n + 1	VIII	VIIIa	. .	3	46:25	VIIId	. .	3	49:22
2n + 1	Total			26	469:216			12	180:80
2n + 2	Total			5	78:27			6	130:43
Total				31				18	

* Aneuploidy preceded by mitotic crossing over.

It is concluded that the centers of these segregants are aneuploids of the hyperdiploid type, trisomic for a single chromosome (2n + 1). The reduced growth rate of the center is due to general unbalance produced by the additional chromosome, rather than gene dosage effect of the mutant present in any one case. This became clear when it was found that aneuploids trisomic for the same linkage groups look exactly the same, if originating from the same diploid regardless of which homologue is present twice (Figures 2b and 3b), and even if originating from differently marked diploids. Aneuploids trisomic for different linkage groups on the other hand show different appearances. Two of the eight 2n + 1 types, trisomic for one of the eight linkage groups are shown in Figures 2 and 3. The replated segregants shown in Figure 2 each originated as an independent event, while the corresponding types in Figure 3 are intermediate segregation products replated from a 2n + 3 segregant (R 133). Figures 2a and 3a show hyperdiploids with an extra homologue of linkage group I (Ib), Figure 2b a trisomic with an extra homologue IIIa, Figure 3b with an extra IIIb. This remarkable phenotypic identification of specific trisomics closely parallels the different recognizable tri-

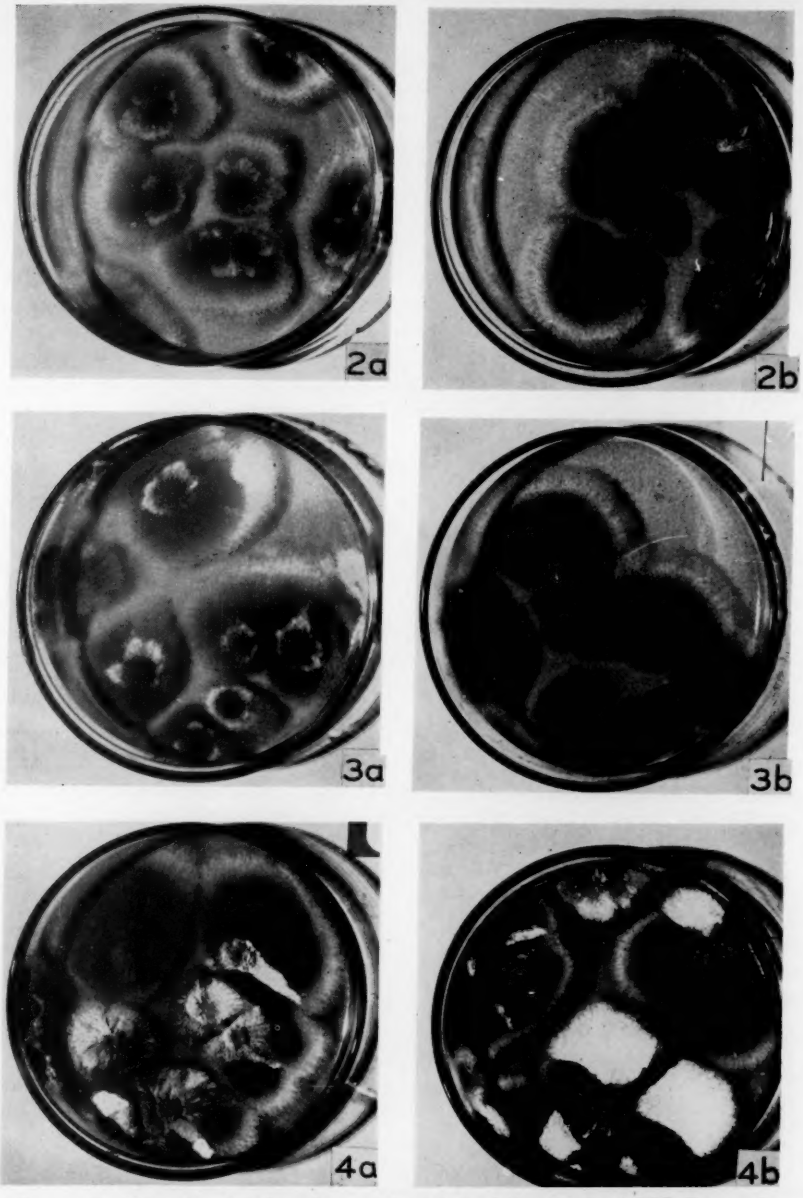


FIGURE 2.—Trisomics of independent origin (a) 2n + Ib; (b) 2n + IIIa. FIGURE 3.—Trisomics isolated as intermediate segregation products from a hyperdiploid (R 133) (a) 2n + Ib; (b) 2n + IIIb. FIGURE 4.—(a) Hyperdiploid R 133, 2n + Ib + IIb + IIIb; (b) Segregant from R 133, 2n + IIb + IIIb.

somic types in various species of higher plants (e.g. in Datura, for which a wide variety of aneuploids have been identified, BLAKESLEE and BELLING 1924).

Abnormal segregants of the types (3) and (4), showing frequent and regular nondisjunctional segregation for markers on two or three chromosomes, are then logically classified as 2n + 2 or 2n + 3. This seems all the more justified since secondary 2n + 1 or 2n + 2 intermediate types were usually recovered from these (all identified ones are listed in Table 4). From a particularly well analysed 2n + 3 segregant (R 133) ten different intermediate types, either 2n + 1 (Figure 3) or 2n + 2 (one shown in Figure 4) were recovered and identified (over 1000 sectors from 11 secondary platings were tested). Four of these intermediate aneuploid types were found to be homozygous for one of the three extra chromosomes, as can be expected to occur in a third of the cases. If care is taken in the collection of conidia from the center of aneuploids, such platings are amazingly uniform, giving mostly centers of the original type and only occasional ones of a type closer to diploid.

It seemed logical, therefore, to conclude that sectoring segregants which show homozygosity for markers of one or two chromosomes (the types 2 and 4) are such intermediate segregation products and that the original aneuploid segregant producing them was trisomic for the corresponding linkage groups. The ploidy of

TABLE 4

Hyperdiploid segregants showing high frequency sectoring for more than one linkage group

Inferred type of original aneuploid	Diploid of origin	Genotype of original aneuploid	Recovered intermediate aneuploid stages	No. of sectors tested
2n + 2	C	2n+Ic+IId	2n+Ic, 2n+IId, 2n(Ic/Ic)+IId	78
	R	2n+Ia+IIIa	2n+Ia	52
	R	2n+IIb+IIIb	2n+IIIb	20
	C	2n+IId+Vd	2n+Vd	23
	R	2n+IIb+VIIa	2n+IIb, 2n(IIb/IIb)+VIIa, 2n(VIIa/VIIa)+IIb	41
	C	2n+IIIc+IV(c-d)*	2n+IIIc	105
	R	2n+IIIa+VIIIb	2n+IIIa, 2n+VIIIb	26
2n + 3	R	2n+Ib+IIb+IIIb	2n+Ib+IIb, 2n+Ib+IIIb, 2n+IIb+IIIb, 2n(IIIb/IIIb)+Ib+IIb, 2n(IIb/IIb)+Ib+IIIb, 2n+Ib, 2n+IIb, 2n+IIIb, 2n(IIb/IIb)+Ib, 2n(IIIb/IIIb)+Ib	1250
2n + 3	Inferred from intermediate recovered stages			
	C	2n+Ic+IId+VIc	2n+VIc, 2n(VIc/VIc)+Ic+IId	21
	C	2n+Ic+IId+VIIc	2n(VIIc/VIIc)+Ic, 2n(VIIc/VIIc)+IId	71
	C	2n+IId+IVd+Vd	2n(IVd/IVd)+IId+Vd	10
	C	2n+IId+VId+VIIId	2n(VId/VId)+IId)+VIIId	18
2n + 4	Inferred from intermediate recovered stages			
	C	2n+IId+IIId+Vd+VIIId	2n+IId+Vd+VIIId, 2n+IIId+Vd	69
	C	2n+IVd+Vd+VIc+VIIc	2n(Vd/Vd, VIc/VIc)+IVd+VIIc	52

* Mitotic crossover chromosome.

the original segregant is therefore inferred correspondingly and, for example, all segregants homozygous for one chromosome, which segregate in the sectors for a second one (type 2), are listed as originally 2n + 2 (Table 3). Similarly, trisomy for three or four chromosomes is inferred in most of the cases listed in Table 4. That their centers were not recovered, can be accounted for by the sparsity of conidia in these higher aneuploid types so that conidia collected for replating are likely to be of a segregant, less abnormal, type.

In three cases many diploid recombinant sectors were found which did not show the expected "nondisjunctional" phenotype. Haploids isolated from these segregants showed that in all cases a mitotic crossing over had preceded aneuploidy and one or two of the trisomic chromosomes were products of a single mitotic exchange. Of the two cases from diploid R the segregant R 26 contained three different homologues of linkage group III, the two original *phen2 s0*$^+$ and *phen2*$^+$ *s0* and a third, a crossover type, *phen2 s0*. It produced frequent diploid sectors, wild type or homozygous for either *phen2* or *s0*, showing apparently high frequency of twin spotting. The segregant R 21 carried three homologues of linkage group II (*Acr*$^+$ *w2 ni3*, *Acr*$^+$ *w2 ni3*$^+$, *Acr w2*$^+$ *ni3*), two of them the reciprocal products of a mitotic exchange, and produced frequent segregant sectors, either homozygous *w2* or *ni3*. The third case, from diploid C, gave *pyro* and + sectors, and contained the two original (*meth pyro*, and *meth*$^+$ *pyro*$^+$) and a crossover (*meth*$^+$ *pyro*) homologue of linkage group IV.

(b) *Unstable types, sectoring without segregation of markers (unmarked chromosomes, diploid C)*: The most difficult types to interpret are unstable segregants which form sectors but do not show segregation for any marker, and are classified as types (5) and (6). Segregants of this type from diploid C may contain an extra chromosome which carries no marker and many show an appearance consistent with this assumption. Their frequency (compared to the corresponding identifiable types) and the fact that three of the total of 12 such cases from diploid C are found to be of type (6), with centers and sectors nondisjunctional for the markers of one chromosome, support this hypothesis.

Similar segregants from diploid R cannot be explained in this way. Most of them also show a very different appearance, namely extremely aconidial centers and relatively small conidating sectors. One of them shows extremely small conidial sectors and resembles very closely a rare type of translocation segregant. It is therefore classified as such. The nine others looked very much alike, all of them with aconidial vigorous centers; one of them was found as a large sector. Detailed analysis in one case did not reveal any change in the genotype. The absence of chromosomal segregation and the origin of these types as sectors calls to mind similar, cytoplasmically determined, sectors found in *Aspergillus glaucus* (SUBAK-SHARPE 1956). Also, acriflavine which has been shown to induce cytoplasmic aconidial types (ROPER 1958) appeared to increase the frequency of such aconidial sectors. But further analysis will be needed, to decide, whether any of these aconidial, sectoring types are in fact cytoplasmic segregants.

Furthermore, among these "abnormals" from diploid R which produce sectors but not segregation for any of the markers there are five cases which did not show

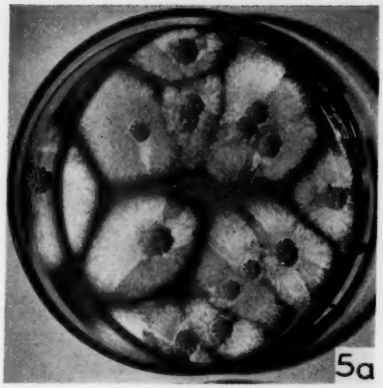
the aconidial appearance; four of them resemble certain hyperdiploid types, but since no photographs were taken, it is not possible to classify them with certainty (all aneuploids are extremely unstable, so that they cannot be transferred onto slants, and are only kept in the original or replated petri plates for a limited time). They might possibly carry one extra chromosome, which has lost its marker by mitotic crossing over. One additional case, which grew well but somewhat irregularly and produced very rare normal looking diploid sectors, showed several changes in the genotype, indicating the occurrence of a spontaneous aberration.

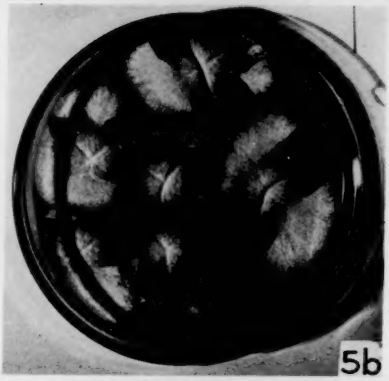
(c) *Hyperhaploids:* Sectoring segregants which give haploid sectors only are classified as hyperhaploids (type 7). In all cases they show the mutant phenotype of several chromosomes, for which they are presumably hemizygous. They always segregate for all other linkage groups, giving in the haploid sectors approximately a 1:1 ratio for the two homologues of each of these disomic linkage groups. Surprisingly few hyperhaploids were recovered (Table 6), but the few types found here correspond exactly to the hyperhaploids isolated with high frequency when conidial heads of mutant color were "needle plated" (KÄFER 1957, 1960). Again the number of extra chromosomes was usually low, four being the highest number of disomic chromosomes found. As found in hyperdiploids, hyperhaploids with increasing numbers of extra chromosomes are increasingly abnormal (Figure 5a–d) with n + 3 producing extremely tiny, practically aconidial centers. And, as found in the case of the 2n + 1, haploids with one extra chromosome have a specific growth type, according to which linkage group is disomic. Figure 5a shows a disomic for linkage group III (which produces sectors of two types, those carrying IIIb lighter than those carrying IIIa) with an appearance very similar to that of trisomics 2n + III (Figures 2b and 3b). Figure 5b shows another n + 1 segregant, disomic for linkage group IV, which forms considerably larger centers.

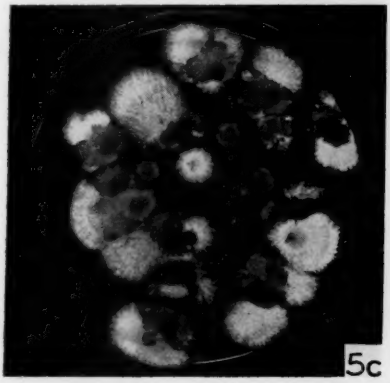
No attempts have been made to determine the frequency of the secondary segregation events in the aneuploids. The size of the aneuploid center and the number of conidia it produces obviously depend to a large extent on the growth rate of the abnormal type as well as on the chance occurrence of an early or late secondary segregation step.

(d) *Breakdown types:* A further type of unstable segregants (type 8) was found which produces a bewildering variety of different nondisjunctional as well as many haploid sectors. One of the four cases from diploid C is given in detail in Table 5b (only the genotypes of stable recovered sectors are listed). To permit simple tabulation of many different types, the symbol of each homologue is used to indicate homozygosity or hemizygosity of all corresponding markers (a or b from diploid R, c or d from diploid C, see Figure 1), while + indicates wild-type phenotype and presumably heterozygosity (? is used where epistasis does not permit distinction of the different types by simple tests).

Two cases from diploid R, which looked very similar, were both recovered as single colonies in secondary platings of hyperdiploid segregants. One of them was found in a replating of a 2n + Ib + IIIb intermediate type from the well analysed







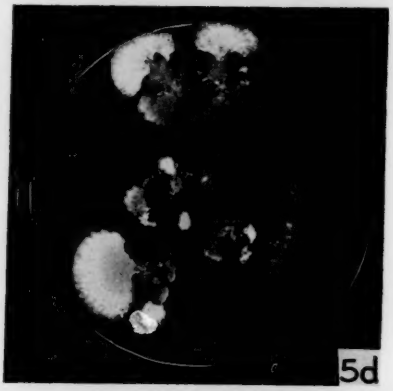


FIGURE 5.—Hyperhaploids: (a) and (b) disomic for one linkage group, (a) n + III; (b) n + IV; (c) n + 2; (d) n + 3.

FIGURE 6.—Breakdown types. (a) R 133.1, original isolate from R 133. (b) R 133.1.13, secondary isolate from R 133.1.

2n + 3 (R 133). The extremely abnormal colony grew to a large size, producing tiny conidiating sectors all over, and only one large, diploid sector was able to outgrow it on one side (segregant R 133.1, see Figure 6a). When conidial heads from different areas were replated, only one second order colony of the same type was recovered (segregant R 133.1.13, Figure 6b). All segregants recovered from both these colonies are listed in Table 5a. The haploids show that at least in R 133.1 all recessive markers were still present. Since only stable 2n and n sectors were isolated by transferring conidia from sectors, needle platings were made to recover possible intermediate steps. While several aneuploids were recovered in this way, it turned out that they all contained the same extra homologue (IIIb) present in the segregant of origin (R 133). This can therefore not be taken as evidence for a stepwise segregation as found in the aneuploids discussed above. In none of these cases was it possible to deduce the genotype from the recovered sectors. Whether a different process is operating, or whether special competitive conditions are present, which might be caused by an unusually high aneuploid chromosome number, is still a matter of speculation.

(e) *Lost unstable types (Table 1):* As mentioned above, extremely abnormal types are often lost, when they form stable sectors very early (type 9) or when normal colonies grow and conidiate freely close by (type 10). In most cases, where sectors but no centers were recovered, these were of at least two types, mostly diploids, nondisjunctional for one or two chromosomes; only in a few cases were haploid sectors present. It seems likely, therefore, that these segregants were highly unstable aneuploids, most of them of a hyperdiploid type. The small number of different sectors recovered did not permit the deduction of their genotypes.

(f) *Translocation segregants (from diploid R)*: A large number of sectoring segregants showed segregation patterns which were of a completely different type. Since in all cases the markers of the two translocation linkage groups (VI and VII) behaved differently from all others, it became obvious that the abnormal type of segregation obtained was due to the structural heterozygosity (type 11). Also, many of these segregants were directly comparable to segregants obtained from diploids heterozygous for another well-analysed translocation (and will therefore be discussed together with these and published elsewhere). These unstable segregants fall phenotypically into three main groups, some showing haploid sectors only, others showing diploid and haploid sectors, and a rare aconidial type giving diploid sectors only.

The observed frequency of such translocation segregants is surprisingly high (a total of 22 among about 9900 colonies from diploid R), but consistent with the hypothesis that these segregants arise mainly by mitotic crossing over in the structurally heterozygous arms of the linkage groups involved in the translocation.

DISCUSSION

The results presented above show that most mitotic segregants obtained from the two diploids studied are easily classified into the four categories of segregants which have been identified in earlier work: (1) diploid mitotic crossovers and

TABLE 5

Phenotype of all recovered sectors from "Breakdown" types

(a) Segregant R 133.1, recovered from plating of R 133 and segregant R 133.1.13, recovered from plating of R 133.1

Ploidy	Color	Linkage groups VIII	I	II	III	IV	V	VI	VII	Numbers 133.1 Transferred	133.1 Plated	133.1.13 Transferred
2n	dark green	a	b	+	+	+	+	+	+	5	.	1
	white	..	..	b	+	+	+	+	+	1	.	.
	dark green	..	..	+	a	+	+	+	a	.	.	4
2n + 1	dark green	..	..	+	+	+	+	+	+/b	.	3	.
n	yellow-chartreuse	b	a	a	a	b	a	a	b	1	1	.
n	white	a	b	b	a	a	a	a	a	2	.	2
		..	..	..	.	..	..	..	b	.	.	1
n + 1	grey	..	..	..	..	..	..	..	+	.	5	.
n	white	..	..	..	..	a	b	b	a	.	.	2
		..	..	..	..	..	..	..	b	1	.	.
		..	..	..	..	b	a	a	a	1	.	.
		..	..	..	..	b	b	b	a	.	.	2
		..	..	..	..	..	..	..	b	1	1	.
		a	b	b	b	a	a	a	b	.	1	.
n	dark green	a	b	a	a	a	a	a	a	.	1	.
		..	..	..	..	a	b	b	a	.	.	1
		..	..	..	..	b	a	a	b	.	.	1
		a	b	a	b	a	b	b	b	.	1	.
n + 1		..	..	..	..	b	b	b	+	.	1	.
n		..	..	..	..	b	a	a	b	.	1	1

(b) Segregant C 22

Ploidy	Color	Linkage groups I	II	III	IV	V	VI	VII	VIII	No.	
2n	green	+	+	+	+	+	+	+	+	19	
		..	..	..	..	..	c	+	+	9	
		..	..	..	..	..	+	+	d	1	
		+	+	+	d	+	+	+	+	1	
		+	+	+	d	+	c	+	+	1	
		+	+	c	+	+	c	+	+	1	
	dark green	d	+	+	+	+	+	+	+	1	
	white	+	d	+	+	+	+	+	+	1	
		..	..	..	..	..	c	+	+	1	35
n	yellow	c	c	d	c	c	c	d	c	1	
		..	..	d	d	c	d	d	c	1	
	white	c	d	d	c	c	c	d	c	1	
		..	..	c	d	c	c	c	c	1	
		..	..	c	d	d	?	d	c	1	
		d	d	d	c	c	c	c	c	1	
		..	..	d	c	c	c	d	d	1	7

Absence of a symbol for any linkage group indicates identity with the one above.

(2) diploids of a nondisjunctional type, (3) haploids and (4) aneuploids. While no more than random coincidence of mitotic crossing over with any of the other three types is found, it is confirmed that there is a connection between the latter three, in so far as aneuploid segregants regularly and with apparently high frequency produce either or both of the other two types, namely nondisjunctional diploid and haploid sectors (KÄFER 1960). At least two, and possibly only two, independent processes are therefore responsible for the appearance of mitotic segregants in *Aspergillus nidulans*: the first is mitotic crossing over, the second a process of chromosomal segregation.

(a) Mitotic crossing over

While the present analysis was designed to obtain information about the second of these processes, some new data on mitotic crossing over have also been obtained, due to the nonselective techniques used here. Mitotic crossing over occurs in the four strand stage of the mitotic division, with the result that two of the four strands are of a recombinant, two of a parental type. Segregation of the centromeres is normal, producing either two reciprocal segregant daughter nuclei each with one crossover and one parental strand, or one parental and one recombinant nucleus, the latter containing both crossover strands. This process was first elucidated by STERN (1936) in his brilliant analysis of somatic recombination in *Drosophila melanogaster*. There, cases of the first type of centromere segregation could be detected in the so-called "twin spots," which consist of two adjacent patches showing reciprocal mutant phenotypes, corresponding to two recessive markers originally in repulsion on the same chromosome arm. Such twin spots cannot be recovered easily with the markers available so far in *A. nidulans*, but in the present analysis two have been found: one showed a crossing over in linkage group I proximal to *paba*, the other in linkage group VIII, proximal to *ribo2*. The other type of centromere segregation, that produces a nucleus containing the two reciprocal crossover strands, has been identified in several cases in Aspergillus, all of them selected as mitotic crossovers between alleles of a single gene (ROPER and PRITCHARD 1955). In the present analysis two cases of a nucleus containing the two reciprocal products of a single mitotic exchange between genes were encountered: one showed a crossing over in linkage group VIII, which occurred very early in the purification of diploid C, the other a crossing over in linkage group II, which led to the formation of unexpected types of sectors in an aneuploid (R 21). These cases show conclusively that, as postulated by PONTECORVO *et al.* (1954) and supported by later evidence (ROPER and PRITCHARD 1955; PONTECORVO and KÄFER 1958), reciprocal mitotic crossing over followed by either of the two normal types of segregation of the centromeres is operating in *Aspergillus nidulans*.

Under standard conditions, in structurally homozygous arms, mitotic crossing over shows a regular and characteristic distribution. Compared to meitotic maps it appears to be relatively more frequent in proximal parts of the chromosome arms (in Drosophila as well as in Aspergillus) and may actually correspond

more closely to the physical length of the chromosomes. Absolute frequencies of mitotic crossing over in different arms, therefore, are not much influenced by the exact position of a fairly distal marker and the average frequency of mitotic crossing over per marked chromosome arm is a meaningful value which may be used to compute the total incidence of mitotic crossing over per mitosis. An average value of 0.07 percent (Table 2) was obtained for the frequency of mitotic crossovers homozygous for a single color marker and of about 0.05 percent for the "requiring" markers, which are expected to be less viable. These frequencies are slightly higher than the values obtained earlier, when an average of 0.04 percent for "requiring" segregants (7800 colonies tested from a diploid with four heterozygous nutritional markers, KÄFER, unpublished) and an average of 0.03 percent of segregants homozygous for a single color marker (*y* or *w*) were detected (PONTECORVO *et al.* 1954). In the two diploids studied here it was found that relatively more segregants were recovered when the density in the original platings was decreased. The difference between the two diploids R and C (Tables 1, 2 and 6), as well as the differences between the present and the earlier experiments, are therefore considered to be due to differences in experimental procedure rather than to strain differences. For the estimate of the over-all incidence of mitotic crossing over in *A. nidulans* (with at least 14 genetically fairly long chromosome arms in the haploid set) a value of about 2 percent is obtained ($14 \times 2 \times 0.07$ percent, assuming that both types of centromere segregation are equally frequent); that is, on the average one in 50 mitotic divisions show such an event.

No more than random coincidence of mitotic crossing over in different chromosomes is observed, as has also been found earlier for coincidence in different intervals of the same chromosome. This is in great contrast to the results obtained when selection is applied within a small segment. Negative interference and/or nonreciprocal exchanges are found to a very large extent in the latter case (PRITCHARD 1960), as has been observed also in yeast (ROMAN and JACOB 1958).

Results from the segregation in diploid R indicate that mitotic crossing over in structurally heterozygous chromosome arms leads to the formation of unbalanced, sectoring types, which look like aneuploids produced by chromosomal segregation. They are presumably monosomic and trisomic for translocated pieces (details will be published elsewhere). The absolute frequency of these translocation segregants was found to be about 0.2 percent (Table 1). This frequency, presumably the frequency of mitotic crossovers homozygous for either one of the three markers on the involved chromosome arms (comparable to the value of 0.05–0.07 percent observed for single markers in other arms) is about half the frequency of all identified aneuploid chromosomal types from the same diploid (0.4 percent, Table 1). This shows how structural heterozygosity, even if of a simple type, can greatly influence the proportion of the different segregant types. Many similar and some more extreme segregation abnormalities have been encountered in the preparation for this analysis, when various irradiated mutant strains were checked for chromosomal aberrations.

The high frequency of very aberrant segregation patterns in the same test diploids (R and C) encountered after treatment of conidia with mutagenic agents

will be discussed in detail elsewhere; for example, after high doses of gamma radiation it is found that the competitive, often haploid, products of segregation show a high coincidence of mitotic crossing over and chromosomal segregation, and many stable products appear to be permanent aneuploids (KÄFER, unpublished). It is not surprising, therefore, that in *Penicillium chrysogenum* results from spontaneous and nitrogen mustard induced segregation differ widely (MUCHNIK 1961). It seems likely that the high frequency of segregation after treatment with agents which produce a high rate of chromosome breaks is only apparent, since nuclei which had lost the damaged chromosomes and contained a haploid set of normal ones would be at a selective advantage and produce sectors. Segregation induced by such agents would therefore be a mechanism of recovery different in each differently damaged cell and not be very useful for the mapping of new markers. More specifically acting substances, however, may perhaps affect one of the segregation processes more directly (e.g. it is claimed that para-fluoro-phenylalanine increases chromosomal segregation only; MORPURGO 1961; LHOAS 1961; the evidence is, however, mostly based on the recovery of haploid sectors, which cannot decide this question).

So far, mitotic crossing over has been identified with certainty only in a very few organisms which show somatic segregation: in addition to Drosophila it is assumed to occur in yeast as a reciprocal process (ROMAN and JACOB 1958). It also seems to occur in the endosperm of maize (MCCLINTOCK 1932; JONES 1937) but other processes producing variegation appear to be much more frequent (MCCLINTOCK 1941, 1956; JONES 1944; BRINK 1958). Some evidence for mitotic crossing over has also been obtained in other fungi: in *Penicillium chrysogenum* (SERMONTI 1957) and in *Aspergillus niger* (HUTCHINSON 1958) and some kind of mitotic exchange seems to occur in the basidiomycete *Schizophyllum commune* (CROWE 1960). In many other cases where variegation occurred, mitotic crossing over does not appear to provide a likely explanation. It seems possible, however, that some cases of regular mitotic crossing over are not recognized because the segregation patterns are distorted in the presence of aberrations. For example a case of an unstable diploid of *Aspergillus nidulans* has been reported by MORPURGO and SERMONTI (1960); it showed sectors produced by mitotic crossing over in linkage group I and an unusual type of segregation in II (interpreted as breakage fusion cycle) which could well be due to the presence of an aberration. Also, the rejection of mitotic crossing over as an explanation of twin spots in tomatoes, on the grounds that no somatic pairing is observed (ROSS and HOLM 1960), does not seem justified until more is known about the relationship of visible pairing and mitotic crossing over.

(b) Chromosomal segregation

The present analysis shows that in *Aspergillus nidulans* segregants of a second type are produced by some kind of chromosomal segregation. It has been postulated that a single process would produce all these "chromosomal" segregants—aneuploids, nondisjunctional diploids and haploids (KÄFER 1960). The present

results support this hypothesis. This process is assumed to be essentially a two step mechanism. The first step happens in the original (heterozygous) diploid: as a rare event of misdistribution of a small number ($x = 1$ or 2) of chromosomes it produces two reciprocal aneuploids, one trisomic the other monosomic for the chromosomes in question ($2n + x$ and $2n - x$). Such a process would be called nondisjunction by definition. The unbalanced types produced would show reduced viabilities and the recovery of the two aneuploids would depend on their abilities to form conidia. The second step would be produced by further (possibly independent) occurrences of the same process in the aneuploid segregants. The chance of such secondary nondisjunction would depend on the number of divisions produced by the aneuploid nucleus in competition (or symbiosis, see below) with the normal nuclei. Wherever a secondary step of segregation would restore the diploid or produce a haploid chromosome number a normally viable segregant would be formed.

This two step mechanism is suggested by the following main observations: (1) That the primary event is likely to be nondisjunction of one or two chromosomes is deduced from the fact that the majority of primary aneuploids from the diploid are of the type $2n + 1$ or $2n + 2$ (56 out of the 78 identified cases, Table 6). The corresponding $2n - 1$ and $2n - 2$ types are not found, however, and further possibilities will be discussed below. (2) The stepwise secondary process of loss of single chromosomes is directly observed in all hyperdiploids and hyperhaploids with more than one extra chromosome and intermediate types are easily recognized and isolated in all cases (for hyperdiploids see Table 4).

(1) *The primary event of chromosomal segregation in the diploid:* Since the frequencies of the different types of aneuploid segregants (Table 6) do not obviously agree with the postulated mechanism of nondisjunction (e.g. no $2n - 1$ and $2n - 2$ are found) the two other most plausible processes of chromosomal segregation which have been found to operate in tissues of higher organisms are also considered here. The most obvious one is complete breakdown of mitosis resulting in random distribution of chromosomes (first discussed as a possible mechanism of haploidisation by PONTECORVO *et al.* 1954). Such a mechanism would produce all different types of aneuploids as well as haploid, triploid and tetraploid segregants. The other is somatic reduction or genome segregation (HUSKINS 1948; GLÄSS 1957; STERN 1958), which produces four haploids or near-haploids from a dividing diploid nucleus. The latter has been briefly discussed earlier (KÄFER 1960) and is not supported by any evidence from the present results. It would mainly produce haploids and hyperhaploids, which were both observed so rarely (among a total of 13,500 conidia from the two diploids R and C two haploids and nine hyperhaploids were found, the latter being about 1/7 of all aneuploids), that this process could not contribute more than a very small fraction of all segregants.

The two main mechanisms which have to be considered for the primary segregation event in the diploid are, therefore, nondisjunction and random distribution of chromosomes. Both would produce the types which were most frequently recovered ($2n + 1$, $2n + 2$) as well as others which were not found (nondisjunc-

TABLE 6

Frequency of the different types of aneuploid segregants

Type			Diploid R		Diploid C	Frequency R	Frequency C
Hyperdiploids							
(a) Identified:							
2n + 1				26	12	..	..
2n + 2	2n + 1, homozygous for 1		5	6		..	..
	2n + 2		4	9	3 9	..	..
2n + 3	2n + 2, homozygous for 1		0	3		..	..
	2n + 3		1	1	2 5	..	..
2n + 4	2n + 2, homozygous for 2		.	.	. 1	..	..
(b) Segregating for unmarked chromosomes				5?	. 12?	..	..
Total				41	39	0.4%	1.1%
Hyperhaploids							
n + 1				2	1	..	..
n + 2				1	1	..	..
n + 3				1	2	..	..
n + 4				0	1	..	..
Total				4	5	0.04%	0.15%
Breakdown types*							
Hyperdiploids (?), giving haploids				2	4	0.02%	0.1%
Total of analysed aneuploids				47	48	0.46%	1.3%

* Several of these were second order segregants.

tion mainly 2n − 1 and 2n − 2, while random distribution would also produce many others). Thus in either case it has to be assumed that some of the types are too inviable to be recovered. The reduction in viability shown by the different aneuploid types recovered supports these assumptions. While the most viable aneuploids, 2n + 1 and n + 1, grow to a fair size (the central part of the colonies in Figures 2, 3, 5a and 5b) before they are outgrown by their respective 2n or n sectors, neither is able to compete when grown in mixture with the sector types. As expected, aneuploids with two or three extra chromosomes grow even more slowly and are very poorly conidiating (see the very central parts in the colonies of Figures 4, 5c and 5d). Even segregants which are probably monosomic for only part of a chromosome show reduced growth rate and conidiation.

The problem of deducing the frequency of events from the observed incidence of certain surviving, permanently changed, products has been excellently discussed by PUCK (1960) in connection with his attempts to measure the dose of radiation which produces an average of one primary chromosome break per cell in tissue cultures of human cells and to compare it with similar earlier observations. In the present case growth rate and especially formation of conidia are the main factors influencing the recorded frequency of the different primary aneuploid products, and recovery depends on especially favorable circumstances (which explains why no aneuploids were recorded among the 13,000 inspected colonies from conidial platings with a higher density than used here, PONTECORVO *et al.* 1954).

The most frequent and fairly viable class of 2n + 1 hyperdiploids is therefore used separately to compare with the predictions of the hypothesis of random distribution of chromosomes. Among the total of 49 recovered 2n + 1 types, 38 were heterozygous for all seven disomic linkage groups and the other 11 were homozygous for one and none for more than one. On the basis of a random distribution of chromosomes, less than one case is expected to be of the first kind and about four of the second, while the rest (44) should be nondisjunctional (homozygous) for more than one linkage group. Thus, there is no likelihood at all that random distribution of chromosomes could produce the observed proportion of the two kinds of 2n + 1 hyperdiploids, nor is this process likely to be responsible for most of the rarer types. However, its rare occurrence cannot be excluded. Nondisjunction, on the other hand, is expected to produce mainly 2n + 1 types which show no segregation for all the disomic linkage groups. Consequently it is assumed that the 2n + 1 types, homozygous for a second linkage group, are not primary products but result from secondary loss of a chromosome in a 2n + 2.

(2) *The secondary process of stepwise loss of chromosomes:* Only a small number of aneuploids other than 2n + 1 and n + 1 have been recovered and identified (14 hyperdiploids and six hyperhaploids, Table 6). All these produce sectors of many different kinds, aneuploid with fewer extra chromosomes and euploid. The former type is still "abnormal," usually fairly small and situated close to the center, and regularly produces euploid sectors at the periphery in a further step of segregation (Figures 4, 5c and 5d). That is, loss of single chromosomes is found in all cases, but it is not possible to decide whether occasionally two or three chromosomes may not be lost in a single event. The regularity and randomness with which one of the three homologues is lost in a 2n + 1 aneuploid is indicated by the almost perfect 2:1 ratio, among the 2n sectors, of wild type (heterozygous):nondisjunctional (homozygous) type (see Table 3). Nondisjunctional diploid types are therefore produced with high frequency from hyperdiploids and in no case has origin from a 2n − 1 been observed.

While there is no doubt that a stepwise loss of single chromosomes occurs in isolated aneuploids, it is not possible to tell whether the process or its frequency are the same in the mycelium of an original colony where diploid and aneuploid nuclei divide in the same cytoplasm. It seems possible that aneuploids show a higher rate of division under such heterokaryotic conditions and certain types are able to divide which are not recovered when conidia are plated. This would explain how haploids could be formed by the stepwise loss of single chromosomes from intermediate products (2n − 1 = n + 7, n + 6 and n + 5) which have never been recovered (contrary to optimistic statements by PONTECORVO in CAVALLI-SFORZA *et al.* 1959).

That most haploids are produced from intermediate aneuploid types has been found consistently in all well analysed diploids. Using the specially favorable method of isolating conidial heads showing mutant color by "needle plating," it was possible to identify such unstable aneuploid precursors in most of the 73 recovered cases of haploids (KÄFER 1960). In the present analysis, however, a

rare type of segregant was recovered which, while being at least 2n, frequently produced haploids without much evidence of intermediate steps (breakdown types, Tables 5 and 6). The segregation seems to be of a chromosomal type, but details of the process or the exact constitution of the breakdown types are not understood. They form a thin, aconidial mycelium which competes extremely well with the numerous conidiating normal segregants it produces (Figure 6), in contrast to hyperdiploids and hyperhaploids, which are regularly outgrown by their sectors.

The mechanism of the secondary process of loss of single chromosomes is assumed to be nondisjunction, but could equally well be lagging of single chromosomes as frequently observed in tissues of higher organisms, or both processes might be operating.

(3) *Frequency of nondisjunction:* It can be concluded that in diploids of *A. nidulans*, in accordance with the present hypothesis, haploids originate in two steps as secondary products from hyperhaploids, and similarly nondisjunctional diploids are formed by the hyperdiploids. The frequency of aneuploid segregants present among conidia of a diploid colony is sufficiently high (at least 1.5 percent, Table 6) to account for the production of all stable haploid (frequency less than 0.02 percent) and nondisjunctional diploid segregants (0.3 percent, extrapolating from the color segregants, Table 2). Thus the incidence of primary nondisjunction is likely to be of the order of two percent and about as high as the incidence of mitotic crossing over; mitotic segregation of either type would occur in *A. nidulans* in at least one out of every 25 mitoses.

Still unsettled is the question whether the frequency of this easily observable secondary process in the aneuploids is any higher than the one of primary nondisjunction in diploids. In view of the extremely different growth rates in these various types it seems impossible to measure and compare such frequencies. It is felt that the main difference is one of competition: missegregation of a chromosome in the 2n produces inferior products, while the reverse is true for all observed aneuploids, since sectors of lower ploidy always conidiate better and outgrow the original. Lagging of chromosomes, if occurring in addition to nondisjunction, would produce a competitive product only in aneuploids; on the other hand, segregation in the aneuploid is of an advantage only if it occurs in the linkage group for which an extra homologue is present.

Only a few rare segregants do not fit into the postulated pattern; some are of an extremely aconidial type (from diploid R) and produce conidial sectors of various sizes but no segregation of markers. One of them has been tentatively classified as a translocation segregant, others which showed no change in the genotype as cytoplasmic segregants. It is hoped that further analysis will clarify their segregation patterns.

(4) *Conclusion:* It appears likely, therefore, that the second main process producing mitotic segregants in diploid *A. nidulans* is a process of misdistribution of single (or few) homologues in mitosis, generally called mitotic nondisjunction. Since hyperdiploids with more than one (usually two) extra chromosomes were relatively frequent (over 20 percent of the hyperdiploids), it must be postulated

that coincidence of nondisjunction in more than one chromosome pair is much higher than random. Similar findings for meiotic nondisjunction in Oenothera, where double nondisjunction seems to be a fairly frequent occurrence, are described by CATCHESIDE (1936) and more than random coincidence was also found by FROST (1961) in Drosophila. Meiosis in *A. nidulans* also produces aneuploids with fairly high frequency (PRITCHARD 1954), but these have not yet been analysed at all. A direct comparison is therefore not possible and further discussion will be restricted to mitotic nondisjunction even though it is felt that the striking parallels in mitotic and meiotic processes (crossing over as well as nondisjunction) may well be due to common features in the underlying mechanisms.

A similar process of mitotic nondisjunction seems to occur in *Penicillium chrysogenum*, where several unstable segregants have been found which give diploid segregant sectors of a single type (the small number of mapped markers made analysis of the responsible mechanism impossible, BALLIO and SERMONTI 1961); these are likely to be hyperdiploids. In higher organisms mitotic abnormalities seem to be more frequent in certain tissues (possibly those with higher ploidy, like endosperm or liver, GLÄSS 1957). It has been suggested that a triploid tissue of a normally diploid organism may show and tolerate a higher frequency of abnormal mitosis and exert less stringent control over the process of mitosis (BRINK and COOPER 1947). The same might then be true for an abnormally diploid strain of Aspergillus, which is a haploid organism. The high frequency of abnormal chromosome distribution in the diploid would then be a phenomenon due to the unusual ploidy (supported by the relatively higher frequency of chromosomal segregation observed among mitotic color segregants in triploids, ELLIOTT 1956, and personal communication). If this is the case, it could not be expected that a similar frequent process of nondisjunction would be operating in tissues of higher organisms.

The main question with respect to the general occurrence of this process of mitotic nondisjunction, however, is the general occurrence of a true mitosis itself. While there seems to be increasing agreement that an unorthodox type of mitosis without a conventional spindle is present in yeasts (ROBINOW 1961), details of mitosis in Neurospora are interpreted very differently by different workers (SOMERS, WAGNER and HSU 1960; BAKERSPIEGEL 1959). No analysis of mitosis in *Aspergillus nidulans* has been carried out so far. Clearly, if the control of vegetative division of nuclei in fungi is of a type different from that in higher organisms, it would also determine the related mechanism of missegregation of chromosomes. Consequently, it is felt that speculations on the actual mechanism will be more profitable when this question is settled (as well as discussions about a suitable term for this type of segregation, for which "mitotic" might be even more misleading than the less specific term "somatic").

Even if a true mitosis is found in Aspergillus and therefore a corresponding mechanism of nondisjunction might be expected in higher organisms, it is likely that its frequency varies, not only between widely different groups of organisms, but even between different strains of the same species as well as between different tissues of the same individual. Such differences would be caused by environ-

mental as well as genetic factors. For example SAX and PASSANO (1961) found that in human cells in tissue culture a nongenetic factor, the age of the culture, was one of the determining factors when the frequency of spontaneous chromosome aberrations was determined, and an interesting case of a single gene controlling the production of mosaic wings in a moth, most likely by loss of chromosomes, has been described by KÜHN (1960).

Differences with respect to the frequency of the various segregant types may therefore be found in either case, whether the processes of mitosis and of nondisjunction are the same or not in any two different organisms which are analysed, usually by different methods.

Very little is known about the process of primary nondisjunction in higher organisms. Aneuploids, on the other hand, are known in many species. Some differences appear to exist between fungi and higher organisms with respect to the frequency of the secondary essentially regulatory events in the aneuploids. While disomics in haploid Neurospora (PITTENGER 1954) and tetrasomics in yeast (COX 1960) are as unstable as aneuploids in *A. nidulans*, trisomics in many higher plants have been found to be exceedingly stable and may even be transmitted through the female gamete (e.g. in Datura, BLAKESLEE 1921; Oenothera, GATES and THOMAS 1914; or maize, MCCLINTOCK and HILL 1930). Similarly many aneuploid strains of human cells in tissue culture seem to show surprising viability and stability, while euploid primary cultures often show increase or decrease by one chromosome even in the first transfer (CAVALLI-SFORZA *et al.* 1959). On the other hand instability of aneuploid types ($2n + 1$) and secondary nondisjunction leading to a more stable 2n type comparable to the process found here, have been postulated to explain human mosaics (GRIBOFF and LAWRENCE 1960). The observed differences and parallels in these results therefore encourage comparison of evidence from different organisms, which will be profitable as long as the possibility is kept in mind that the same results may be produced by different mechanisms, as well as that different results are produced by the same mechanism under different conditions.

SUMMARY

Most mitotic segregants recovered under optimal conditions from two well-marked diploids of *Aspergillus nidulans* appear to be produced by two main processes: (1) mitotic crossing over and (2) a process of misdistribution of small numbers of chromosomes (mitotic nondisjunction). Both processes seem to be about equally frequent and mitotic segregation of either kind is estimated to occur in at least one out of every 25 vegetative divisions.

(1) Mitotic crossovers were found to be about twice as frequent as previously reported. The complementary products of a single event were recovered in four cases, twice in a single nucleus, twice as twin spots. One of the analysed strains was a translocation heterozygote which produced 0.2 percent unstable segregants of a specific type, probably products of mitotic crossing over in the structurally heterozygous arms.

(2) Of the "chromosomal" segregants, unstable aneuploid types showed the

highest frequency (1–2%). Most of them were hyperdiploid, hyperhaploids being found only rarely. The most frequent types were $2n + 1$; they showed a recognizable phenotype characteristic for each trisomic linkage group. All aneuploids are unstable, that is, they are always outgrown by the rare, more vigorous diploid or haploid sectors which they regularly produce. The sectors from a $2n + 1$ are in ⅔ of the cases of parental, in ⅓ of nondisjunctional type with respect to the trisomic linkage group. Hyperdiploids as well as hyperhaploids show a stepwise loss of the extra chromosomes, presumably by the same process that produces aneuploids from diploids (but lagging of chromosomes could equally be the cause). Nondisjunctional diploid segregants are estimated to have a total frequency of about 0.3 percent, haploids of less than 0.02 percent. A few unstable segregants seem to be produced by other processes, some possibly by cytoplasmic segregation.

ACKNOWLEDGMENTS

I wish to express my thanks to Drs. E. R. Boothroyd and F. C. Fraser for their valuable comments and criticism of the manuscript. The technical assistance of K. A. Constantinos is gratefully acknowledged.

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THE BEHAVIOR IN LABORATORY POPULATIONS OF GENES ASSOCIATED WITH TUMOROUS HEAD EXPRESSION IN DROSOPHILA MELANOGASTER[1]

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Received July 14, 1961

IT was first noted in 1945 that a strain of flies, collected originally at Acahuizotla, Mexico in 1941, expressed a head abnormality now known as tumorous head (*tu-h*). GARDNER and WOOLF (1949) determined that the trait was controlled by two genes. One (*tu-1*) is recessive, sex linked, and associated with a maternal effect (GARDNER 1959). The other (*tu-3*) is semidominant, located in the third chromosome, and necessary for the expression of the trait.

Gene *tu-1*, or one of several alleles of this gene, is apparently widespread in laboratory stocks and in natural populations. GARDNER and STOTT (1951) made reciprocal crosses with *tu-h* and 22 "wild" laboratory stocks. A strong maternal effect (GARDNER and WOOLF 1949; GARDNER and GARDNER 1953) was detected from three (Oregon-R, Crimea, and Stephenville) and a less pronounced effect was indicated from one (Amherst-34). Stephenville, collected from the wild at Stephenville, Texas in 1938, was found to have a much greater maternal effect than *tu-h*. An allele of *tu-1* (*tu-1*^{s}, GARDNER and STOTT 1951) was homozygous in this laboratory stock, but no phenotypic effect was detected. When the other gene (*tu-3*) was introduced experimentally the tumorous head expression was observed in a high proportion of the flies. The genes *tu-1* and *tu-1*^{s} are isoalleles in regard to the maternal effect they control. Just as Canton and Oregon wild types differ in amount of eye pigment because of isoalleles at the white locus (GREEN 1959), the *tu-1* alleles affect the degree of maternal effect. Several laboratory stocks carrying mutant genes and collections from wild populations (GARDNER, STOTT and DEARDEN 1952; GARDNER and GARDNER 1953) were found to carry *tu-1* or alleles of *tu-1*. The genes were not homozygous but were represented at different frequency levels in the populations sampled. Because these alleles that have no visible effect on the flies seem to maintain themselves and perhaps increase in laboratory and wild populations, their behavior in populations has become a problem for investigation.

It was found (WOOLF and PHELPS 1960) that the *tu-h* stock had two types of third chromosomes. One (IIIB) contained a homozygous lethal inversion in the left arm. The inversion was identified as the Payne inversion and is associated in the *tu-h* stock with the scarlet eye color gene (*st*). In the second type of chro-

[1] This work was supported by Grant DRG-178F from the Damon Runyon Memorial Fund for Cancer Research and Grant No. P-22B from the American Cancer Society.

mosome (IIIA) this inversion is absent. Inversion heterozygotes (IIIA/IIIB) have a selective advantage that is apparently associated in some way with the inversion.

MATERIALS AND METHODS

Flies used in the experiments were taken from laboratory stocks of *Drosophila melanogaster*. The *tu-h* stock has been closely inbred and selected at Utah State University for the past 12 years. Stephenville, carrying an allele (*tu-1*^{s}) of the gene *tu-1*, and Canton with the wild-type allele ($+^{tu-1}$) have also been maintained in the same laboratory for the same period of time. Samarkand wild stock, also carrying $+^{tu-1}$, was obtained from the University of California in 1957. The stock symbolized *tu-1*^{s} was synthesized by a series of crosses involving marked chromosomes and inbred for several generations. This stock is made up of flies homozygous for *tu-1*^{s} and *tu-3*.

Experiments were carried out in population cages at room temperature. Three of the cages (numbers 1, 4 and 5) were similar to those described by WRIGHT and DOBZHANSKY (1946). Cage 1, constructed of wood with a glass top and screen sides, was $17 \times 13 \times 5\frac{1}{2}$ inches. Cages 4 and 5 were of similar dimensions but sheet metal was used in place of wood, and the top as well as the sides were made of screen. Food containers were 1-oz., screw-cap jars placed on No. 12 stoppers with staples and elastic bands to hold them upright. Cages 2 and 3 were constructed from polyestrene boxes $12 \times 5 \times 5$ inches with ten food vials inserted into the sides. The food vials were 25×100 mm shell vials positioned horizontally. In populations 1, 4, and 5 a fresh food container was added every three days on a cycle of 45 days for a complete rotation. The food was a standard cornmeal, molasses, and agar preparation to which live yeast was added. Genotypes of the flies that were used to initiate the populations are indicated in Table 1. Initial

TABLE 1

Genotypes of parent flies from which populations were initiated

Population	Cross	Genotypes of parents*			
1	*tu-h* × *tu-h*	$\frac{tu\text{-}1}{tu\text{-}1}$	$\frac{tu\text{-}3}{tu\text{-}3}$ ×	$\frac{tu\text{-}1}{/}$	$\frac{tu\text{-}3}{tu\text{-}3}$
2	Stephenville × *tu-h*	$\frac{tu\text{-}1^s}{tu\text{-}1^s}$	$\frac{+^{tu-3}}{+^{tu-3}}$ ×	$\frac{tu\text{-}1}{/}$	$\frac{tu\text{-}3}{tu\text{-}3}$
3	*tu-1*^{s} × *tu-1*^{s}	$\frac{tu\text{-}1^s}{tu\text{-}1^s}$	$\frac{tu\text{-}3}{tu\text{-}3}$ ×	$\frac{tu\text{-}1^s}{/}$	$\frac{tu\text{-}3}{tu\text{-}3}$
4	Stephenville × Samarkand	$\frac{tu\text{-}1^s}{tu\text{-}1^s}$	$\frac{+^{tu-3}}{+^{tu-3}}$ ×	$\frac{+^{tu-1}}{/}$	$\frac{+^{tu-3}}{+^{tu-3}}$
5	Stephenville × Canton	$\frac{tu\text{-}1^s}{tu\text{-}1^s}$	$\frac{+^{tu-3}}{+^{tu-3}}$ ×	$\frac{+^{tu-1}}{/}$	$\frac{+^{tu-3}}{+^{tu-3}}$

* Female always written first.

crosses were started in vials, and the F_1 flies from each pair mating were transferred to the appropriate cage.

Gene combinations in cages 1, 2, and 3 were designed to determine whether a population containing the *tu-1*^{s} allele would produce a higher proportion of flies expressing the tumorous head phenotype than a population containing the *tu-1* allele. At monthly intervals the cages were sampled by extracting flies from each cage with a vacuum apparatus. Flies making up a sample were etherized and examined with the aid of a binocular microscope at a magnification of 45× to determine the percentage of flies expressing the tumorous head phenotype. A total of 101 flies was included in each sample. Following examination, the flies were allowed to recover from etherization and were returned to the cage from which they had been taken.

Cages 4 and 5 were sampled at various intervals to determine the frequency of the *tu-1*^{s} allele in the populations. This was accomplished by extracting female flies from each cage, artificially virginizing the females by cold shocks (NOVITSKI and RUSH 1948), and pair mating them with *tu-h* males. Expression of the tumorous head phenotype among the F_1 flies indicated that the female was homozygous for the *tu-1*^{s} allele. The proportion of heterozygotes was determined by mating F_1 females from the parent matings which had not produced the tumorous head expression, back to *tu-h* males. A heterozygous female from the cage would be expected to produce heterozygotes for the gene that controlled the maternal effect with equal frequency in the F_1. Six F_1 females from each cross were pair mated with *tu-h* males and the progeny were examined for the tumorous head trait. Expression in the progeny from any one mating was interpreted to indicate that the original female from the cage was heterozygous. The probability of no tumorous head expression from all six matings if the original female was heterozygous would be approximately 1/64.

EXPERIMENTAL RESULTS

Results of monthly samplings of populations 1, 2, and 3 are shown in Figure 1. The percentage of flies with the tumorous head expression in the samples from population 1 varied from a high of 89 percent three months after the population was started to a low of one percent in the thirteenth month. In the ninth month of the experiment, males taken at random from the cage were pair mated with scarlet-eyed females. Occurrence of the scarlet eye phenotype among the progeny of a particular mating was interpreted to indicate that the *tu-h* male from the cage contained the third chromosome inversion which includes the recessive gene (*st*) for scarlet eyes. Of 135 pair matings, 105 (78 percent) expressed the scarlet eye phenotype.

Percentages of flies expressing the trait in the samples from population 2 ranged from 19 to 37, with a mean of 28 percent over a 15-month period. Pair matings between males from this population and Stephenville females were made to detect the presence of the *tu-3* gene in the males. In matings that produced progeny expressing the tumorous head phenotype, the male possessed at least

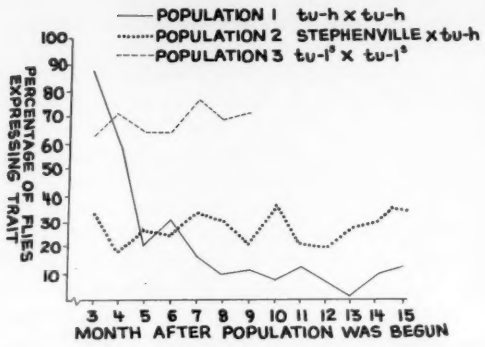

FIGURE 1.—Percentage of flies expressing tumorous head phenotype in population cages measured at monthly intervals.

one *tu-3* gene. From 35 such matings, 15 (42 percent) produced progeny expressing the tumorous head phenotype.

The range of flies expressing the trait in samples from population 3 over a 9-month period was from 64 to 76 percent, with a mean of 68 percent tumorous head flies. Population 3 became contaminated after the ninth month and later samples indicated a change in gene frequency rather than in phenotype selection.

The initial frequency of the *tu-1*^{s} allele in population 4 was .67. The maximum likelihood estimate of the frequency of the allele in a sample taken from the population in the fifth month was $\hat{p} = .32$, with a .95 confidence interval of .22–.65. The estimate from a sample at eight months was $\hat{p} = .30$ with a confidence interval of .20–.50. A sample at 20 months showed $\hat{p} = .18$, with a confidence interval of .08–.37.

Population 5 was sampled for the frequency of homozygous *tu-1*^{s} females only. A sample at two months showed 34 percent homozygous, with a .95 confidence interval of .21–.55. A sample at six months was 15 percent homozygous with a .09–.27 confidence interval. A sample at 17 months was eight percent homozygous with a .01–.23 confidence interval.

DISCUSSION

The level of tumorous head expression in populations 1, 2, and 3 was in proportion to the number of *tu-1* isoalleles present initially. The *tu-1*^{s}/*tu-1*^{s} combination had the highest percentage, with the *tu-1*^{s}/*tu-1* combination at an intermediate level after six months from the time of the first sampling. Regression analysis indicated that the slope of the regression lines for the samples from populations 2 and 3 was not significantly different from zero. The samples from population 1, however, behaved quite differently from the others.

An additional population cage was started using flies of the same genotype as population 1 and was kept for eight months before contamination occurred. Levels of the percentage of tumorous head flies from samples of this population

followed the same type of curve as samples from population 1. Regression analysis of the curve representing population 1 had significant linear, quadratic, and cubic terms at the .01 level of significance. The dispersal of modifiers in population 1 may explain the difference in behavior between populations. Flies used to start population 1 were from *tu-h* laboratory cultures that had been inbred for many generations and had developed progressively increasing proportions of tumorous head flies. It was presumed that this increase occurred because modifiers favoring the tumorous head expression were being accumulated by selection. Modifiers that had not become homozygous would be dispersed through random mating in the population cages where there was no artificial selection. Population 2 was essentially an outcross, whereas population 3 was synthesized from Stephenville and *tu-h* stocks by a series of crosses in which marked chromosomes were introduced. Thus, populations 2 and 3 would not be expected to have accumulated an appreciable number of modifiers favoring the tumorous head expression.

Selection in the three populations was presumably based on phenotype. In culture bottles *tu-h* flies were found to be less viable than those of wild stocks (GARDNER and RATTY 1952). The populations were homogeneous with respect to *tu-1* and *tu-3* and individuals with severe abnormalities were apparently selected against. Levels of expression were not as high in the population cages as in the culture bottles where approximately 90 percent of the flies expressed the trait.

The third chromosome inversion heterozygote had a selective advantage in population 1 as indicated by the frequency of heterozygotes (78 percent in the sample studied). Because the inversion is homozygous lethal, the selection equations have a unique solution: $s = .64$ for noninversion homozygotes. Heterosis has been shown to be favored in laboratory populations (DOBZHANSKY and LEVENE 1951), and it increases the fitness of the population (CARSON 1958). With this premise the maintenance of the inversion system in the population might be expected.

Data concerning the frequency of the $tu\text{-}1^s$ allele in populations 4 and 5 indicate that the allele was not strongly selected for or against in relation to $+^{tu-1}$ alleles from the two wild stocks. There seemed to be a slow decrease in the frequency of $tu\text{-}1^s$ over the total period, but the confidence intervals of the samples overlapped and the decrease was not established. It might be expected that the heterozygotes would increase in proportion to the homozygotes (WALLACE 1958), but because of the indirect method of detecting heterozygotes involving six pair matings it was technically impossible to increase the sample size enough to narrow the confidence limits of the proportion.

The homozygosity of the $tu\text{-}1^s$ allele in the Stephenville, $tu\text{-}1^c$ in Crimea, and $tu\text{-}1^o$ in Oregon-R (GARDNER and STOTT 1951) laboratory stocks, all of which were taken from a wild population, could have been caused by ecological conditions in nature or by environmental situations present in the culture bottles. The same explanation would probably apply to Amherst-34 but complete homozygosity has not been established for this stock. Another possible explanation that seems more likely for all of these stocks is that the natural population might have been small enough at some times, or the method of transferring stocks in the

laboratory might have been favorable for genetic drift to fix an allele already present in the population. Genetic drift would be expected to occur in the common transfer of laboratory stocks. Usually only a few individuals picked more or less at random from a particular stock culture are transferred into a new culture bottle to serve as parents of the next generation. The probability of a certain allele becoming fixed under these conditions is quite high (KERR and WRIGHT 1954).

SUMMARY

1. Experimental populations containing the *tu-1*^{s} isoallele had a greater proportion of tumorous head flies than those containing only *tu-1*, presumably because *tu-1*^{s} has a more pronounced maternal effect than *tu-1*.

2. The data do not indicate why the Stephenville laboratory stock (collected originally at Stephenville, Texas) or the Crimea or the Oregon-R stocks also collected originally from wild populations were homozygous for a *tu-1* allele, but genetic drift is suggested as a possible explanation.

3. In an experimental population, the frequency of a third chromosome inversion increased from 25 or 50 percent (depending on whether one or both parents carried the inversion) to 78 percent over a period of nine months. In heterozygous condition the inversion must have a heterotic effect to account for its perpetuation in the population.

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THE MAPPING OF GENETIC LOCI AFFECTING STREPTOMYCIN RESISTANCE IN PNEUMOCOCCUS[1]

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Received July 28, 1961

THE term "complex locus" implies a series of closely linked genetic determinants all of which produce similar or identical modifications in the phenotype of an organism. Much of the experimental work to be described is concerned with a series of this type conferring streptomycin resistance in Pneumococcus.

BRYAN (1961) has isolated spontaneous streptomycin-resistant mutants of Pneumococcus which show a wide variation in the maximum concentration of streptomycin they are able to resist. A small random sample (four) of these spontaneous mutations was tested for linkage in transformation studies, and they were all found, by the criterion of genetic recombination, to be either allelic (at the same site) or closely linked (at different sites). While this finding could have been due to the small number of mutants tested, it suggested that all streptomycin-resistant mutations occur in the same region (a cistron?) of a DNA molecule. However, analysis of additional markers was necessary to obtain a more complete picture. With this aim in mind the present studies have been undertaken; they have shown that the remainder of the spontaneous streptomycin-resistant mutations isolated by BRYAN (1961), as well as two spontaneous mutations originally isolated by SCHAEFFER (1956), represent either allelic or closely linked markers on the same molecule of transforming DNA. In addition, a nitrous acid-induced mutation to streptomycin resistance has been examined. This mutation appears to belong to a different locus; it does not exhibit the degree of linkage to the spontaneous mutations that the latter exhibit to each other.

MATERIALS AND METHODS

Strains: Two closely related strains of pneumococcus were used in these experiments, SIII-1 and clone 3 of strain R36A. The first was synthesized by EPHRUSSI-

[1] Part of the material in this paper is taken from a thesis (by M.B.R.) submitted in partial fulfillment of the requirements for the degree Doctor of Philosophy, at the University of Rochester, Rochester, New York.

[2] Recipient of a predoctoral fellowship of the National Cancer Institute, U. S. Public Health Service, 1958–1960. The research conducted at Rochester also benefitted by Research Grant E-727 awarded (to A.W.R.) by the National Institute of Allergy and Infectious Diseases.

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TAYLOR (1951a) and produces only traces of type III polysaccharide. The second is a clone derived by single-colony isolation from R36A. The exact relationship of strain R36A to strain SIII-1 is described by RAVIN (1959).

Genetic markers: The streptomycin-resistance markers used in these experiments are derived from four sources. The first is a single-step, spontaneous mutation to high level resistance (6000 μg/ml) isolated in strain SIII-1 by RAVIN (1956). This marker will be referred to as *str-r1* (BRYAN 1961). The second group of streptomycin-resistance mutations used in these experiments was obtained as single-step, spontaneous mutations isolated in strain SIII-1 by BRYAN (1961). The designation of these mutations and their approximate levels of resistance in strain SIII-1, as determined by the criteria of BRYAN, are as follows: *str-r2* (300 μg/ml), *str-r3* (150 μg/ml), *str-r4* (300 μg/ml), *str-r5* (300 μg/ml), *str-r9* (2000 μg/ml), *str-r25* (9000 μg/ml), *str-r26* (10–12,000 μg/ml), *str-r27* (6000 μg/ml). The third group consists of two spontaneous mutations to streptomycin resistance isolated in strain R36A by SCHAEFFER (1956). SCHAEFFER has called these two mutations *r2* and *r3*, resistant respectively to 100 μg/ml and 3000 μg/ml streptomycin. According to the system of nomenclature adopted in this laboratory these will be referred to as *str-r28* and *str-r29* respectively. Most of the markers from these three sources were obtained by selection of mutants at streptomycin concentrations greater than 50 μg/ml. A third mutant which can resist, at most, 25 μg streptomycin per ml was obtained by SCHAEFFER (1956) by selecting at an antibiotic concentration of 12 μg/ml. Unfortunately, this mutant, termed *r1* by SCHAEFFER but redesignated *str-r30* in our nomenclature, was not available for study by us.

The fourth source of markers is a number of mutations induced in clone 3 by nitrous acid using the method described by LITMAN and EPHRUSSI-TAYLOR (1959). The authors are grateful to G. BALASSA who originally isolated and supplied us with these mutants. They confer a relatively low level of resistance (ca. 30 μg/ml) in clone 3.

It should be pointed out that there appears to be a difference in the residual genotypes of clone 3 and SIII-1. While both strains have about the same sensitivity to streptomycin (10^7 cells of either strain failing to produce colonies when plated in concentrations of streptomycin higher than 30 μg/ml), the spontaneous mutations confer somewhat higher resistances in clone 3 than in SIII-1. For example, *str-r3*, *str-r2* and *str-r1* confer resistance to 400, 800 and 20,000 μg/ml respectively, when transferred into clone 3. These differences are probably due to the presence, in clone 3, of enhancer genes of the kind described by BRYAN (1961).

The erythromycin-resistance marker (*ery-r2*) used in these experiments was isolated as a spontaneous mutation to low level resistance (1 μg/ml) in strain Rx (GREEN 1959; RAVIN and IYER 1961). The *ery-r2* marker was introduced in the donor DNA containing a streptomycin-resistance marker to be tested in order to provide a control on the transformability of the recipient strains.

A sensitive strain will be designated as follows: SIII-1 *ery-s2 str-s1*, or *ery-s2 str-s2*, or etc., depending on the streptomycin-resistance marker being discussed.

Media: In Rochester all strains were maintained and transformed in Medium 3 of EPHRUSSI-TAYLOR (1951b). Medium 1 (EPHRUSSI-TAYLOR 1951b) was used for the growth of large populations from which DNA transforming preparations (TPs) were made. Medium 2 (EPHRUSSI-TAYLOR 1951b) was used for assay of transformants. In Gif, transformations were carried out in a modification of Medium 3, called "NS." This medium is essentially similar to Medium 3, but lacking extract of beef serum.

Preparation of DNA: Most of the DNA transforming preparations (TPs) were made according to the procedure described by EPHRUSSI-TAYLOR (1951b). However, the design of some of the experiments demanded that many preparations be made from a large number of independently-derived transformants; to facilitate such studies the procedure was modified to include only one deproteinization of the alcohol-insoluble fraction of the original crude lysate, according to the methods of SEVAG (1934), followed directly by precipitation of the DNA as fibers by the gradual addition of alcohol. These DNA preparations are contaminated with protein and RNA, but since the genetic purposes to which they are put are not concerned with the purity of the DNA preparations, they are suitable for use in these experiments. The DNA preparations were stored either in physiological saline (0.86% NaCl) or buffered saline (0.15 M NaCl, 0.01 M $PO_4\equiv$) at 5°C.

Other DNA preparations were prepared by lysis with versene. Two hundred ml cultures of desired turbidity in Medium 1 were first centrifuged at 1800 rpm for 30 minutes and then resuspended in 5 ml of 0.15 M NaCl, which was 0.1 M with respect to versene, buffered at *p*H 7.6. The suspension was incubated at 37°C for one hour, and lysis was completed after 24 hours at 0°C.

Transformation procedure: Recipient cells are rendered competent by growth in Medium 3 or "NS" to which bovine serum albumin (Armour Fraction IV) has been added to give a final concentration of 0.2 percent. In general, in experiments involving single *str-r* markers, a transforming procedure was used in which a small inoculum of cells was allowed to become competent in the presence of the transforming DNA, and then plated approximately 10–12 hours after competence and absorption of DNA had occurred (RAVIN 1959). This type of transformation procedure is designated as "long term."

Transformation experiments involving more than one *str-r* marker were carried out by a second method designated as "short term." In this method, competent cells are exposed to transforming DNA for 20 minutes (less than one generation time), and the action of the DNA is terminated by a fiftyfold dilution in medium lacking DNA. The treated cells are allowed to grow at 37°C for two hours in order for the transformed phenotype to be expressed (GREEN 1959; RAVIN and IYER 1961). Both the long and short-term experiments were performed with saturating concentrations of DNA.

Controls were cultures of cells grown under conditions identical to the experimental cultures but without the presence of the transforming DNA; such cultures were used to determine the level of resistance of untransformed recipient cells in allelism tests.

Assay of transformants: In most of the experiments transformants were assayed by spreading an aliquot of the culture over the surface of solidified Medium 2 which had been previously dried for 10–12 hours by storage at 37°C. Several aliquots were tested by spreading on each of a number of agar plates containing different concentrations of antibiotic. After spreading, all plates were incubated at 37° for 24–48 hours before counting the number of colonies that developed. In some experiments, transformants were assayed by pipetting suitable aliquots of the culture into Petri dishes and then mixing with molten agar containing the desired concentration of streptomycin. The colonies developing in the agar appeared after 48 hours of incubation at 37°C, at which time they were counted. Either of these methods of determining the level (or levels) of resistance of a transformant population is referred to in the text as that of "direct plating."

To assay the proportion of erythromycin-resistant cells, erythromycin lactobionate (Abbott) was added to Medium 2 at a concentration of 0.25 μg/ml. The proportion of streptomycin-resistant cells was determined by the addition of streptomycin sulfate (Lilly) to Medium 2, the final concentration varying according to the markers used.

In those experiments in which it was necessary to determine the maximum level of resistance of the transformant class, an additional procedure was used. In order for all of the cells to express the maximum level of resistance conferred by a marker recently acquired by transformation, more time must elapse than that required for expression of a lower level of resistance. Therefore, in the direct plating method streptomycin-resistant transformants are selected by exposure to a concentration of streptomycin that is much lower than the maximum concentration they are genetically capable of resisting. The maximum level of resistance of these transformants is determined in one of the following ways. In the case of transformant colonies growing on the surface of agar, a velvet replica is made (Lederberg and Lederberg 1952) of the plate containing the lowest concentration of antibiotic (to insure recovery of all transformants) and restamped on a series of plates of increasing concentrations of streptomycin. In this way a growth-limiting concentration of streptomycin is determined. In the case of transformant colonies growing in agar, the colonies are first isolated by means of a platinum needle to a sector of an agar plate devoid of antibiotic. Plates containing such isolates are incubated to permit a sufficient amount of growth, and the growth in each sector is tested either by the velvet-replica technique or by directly streaking a platinum loopful to each of a series of agar plates of increasing concentration of streptomycin.

That concentration of antibiotic above which a significant fraction of a mutant or transformed strain is unable to grow, varies to a certain extent with the physiological state of the culture (Bryan 1961; Ravin and Iyer 1961). The greatest degree of variation is observed with those mutants capable of resisting the highest concentration of streptomycin. For these reasons, two mutants cannot be phenotypically distinguished if their levels of resistance, although not identical, are very close.

Allelism tests: Two streptomycin-resistance markers will be designated as allelic if they can replace one another but cannot coexist in the same genome, and conversely, they will be designated as nonallelic if they can coexist in the same genome. It has been observed that when nonallelic antibiotic-resistance markers are simultaneously present the level of resistance of the cell is generally increased compared to the level of resistance of the cell when just one marker is present. Furthermore, the new level of resistance is greater than the sum of the levels of resistance conferred by the markers individually (HOTCHKISS and EVANS 1958; RAVIN and IYER 1961; BRYAN 1961). A TP prepared from such a multiply-marked strain will give rise to several classes of transformants. One class is characterized by the same level of resistance as that of the donor strain, due to the sensitive recipient cell acquiring both markers, and the other classes are characterized by lower levels representing acquisition of single markers. The frequency of the different classes is a function of the degree of linkage of the individual markers.

Allelism tests to be described are based on this principle. Recipient strains bearing one streptomycin-resistance marker, e.g. *str-r2*, are treated with DNA of another marker, e.g. *str-r1*. The transformants are then screened for a class (presumably *str-r1-r2*) whose resistance is greater than that of either component or as great as *str-r1* (representing a substitution of the *str-r2* marker by the *str-r1* marker), or even for phenotypes having a level of resistance greater than *str-r2* but less than *str-r1*. Such a class might represent the *str-r1-r2* recombinant since it has been observed (RAVIN and IYER 1961) that nonallelic erythromycin-resistance markers can be antagonistic in their effect on the phenotype. DNA preparations are made from all transformants isolated from such "crosses" and their genotype is determined by treating the sensitive strain with these DNAs, and observing the classes of transformants thus obtained.

Isolation of transformants: Transformants are isolated by restreaking, by means of a platinum needle, single colonies arising in direct platings. The term "independent transformants" refers to colonies selected from different experiments, e.g. five independent transformants indicates that the experiment was done five times; from each experiment a single colony was selected to be tested. In this manner, all independent transformants represent the results of different transformation events.

RESULTS

The sites of str-r2, str-r3 *and* str-r1: Strains *str-r2* and *str-r3* were individually treated with TP *ery-r2 str-r1*. In each case transformant colonies were selected on a concentration of streptomycin at which the recipient cells would not be able to grow (500 or 1000 μg/ml). The results of several typical experiments are summarized in Table 1A, B. The plates containing the lowest concentration of streptomycin were replicated to plates containing concentrations of streptomycin greater than 1000 μg/ml, and in all cases it was observed that the transformants exhibited a phenotype indistinguishable from that of cells bearing the marker *str-r1* alone. Independent transformants were isolated, grown in the absence of

TABLE 1

Allelism tests between different str-r *markers*

Cells	TP	Frequency of transformants ($\times 10^{-4}$)* resisting: Erythromycin 0.25 μg/ml	Streptomycin 500 μg/ml	Streptomycin 1000 μg/ml
A. *str-r2 ery-s2*	*str-r1 ery-r2*	30	–†	9.8
	str-r1 ery-r2	5.0	–	1.0
	none	$< 1 \times 10^{-7}$	–	2×10^{-6}
	none	$< 1 \times 10^{-7}$	–	1.7×10^{-6}
B. *str-r3 ery-s2*	*str-r1 ery-r2*	1.2	.08	–
	str-r1 ery-r2	1.0	.4	–
	none	$< 1 \times 10^{-8}$	4×10^{-7}	–
	none	$< 1 \times 10^{-8}$	4×10^{-7}	–
C. *str-r5 ery-s2*	*str-r2 ery-r2*	1.4	–	4×10^{-7}
	none	$< 1 \times 10^{-6}$	–	6×10^{-7}
D. *str-r28 ery-s2*	*str-r2 ery-r2*	4.7	7×10^{-7}	–
	none	$< 1 \times 10^{-7}$	7.2×10^{-7}	–

* Unless otherwise indicated.
† Hyphen indicates not tested by direct plating.
In A and B all transformants selected on the concentration of streptomycin indicated were able to grow on concentrations of streptomycin up to 6000 μg/ml, as determined by velvet-replica method. In A and B duplicate experiments are reported.

streptomycin for a short time, and their level of resistance determined by means of direct plating and velvet-replica technique. The results confirmed the previous observation of a single class of transformants, phenotypically identical to mutants containing the marker *str-r1*. If the recombinant classes *str-r1-r2* or *str-r1-r3* existed among the transformants, they did not have a greater level of streptomycin resistance than the class expected from a substitution of the *str-r2* or *str-r3* markers by the *str-r1* marker. DNA preparations were made from five independent transformants obtained in each "cross." When the sensitive SIII-1 strain (*str-s1-s2-s3 ery-s2*) was treated with each of these TPs, it was observed (Table 2A, B) by direct plating followed by velvet replication of plates containing the lowest concentration of streptomycin, that only one class of transformants could be obtained. In every case, this class is phenotypically identical to *str-r1*. There is no evidence of the simultaneous presence of the *str-r2* or *str-r3* marker with the *str-r1* marker in the cell lines derived by the treatment of *str-r2* or *str-r3* with TP *str-r1*.

This observation excludes the possibility that marker *str-r1* is unlinked to *str-r2* or *str-r3*. Marker *str-r1* is either allelic to *str-r2* and *str-r3*, or if it is possible to obtain the recombinant classes *str-r1-r3* and *str-r1-r2*, these classes occur less frequently than once in about five recombination events in the "crosses" *str-s1-r2* × *str-r1-s2* and *str-s1-r3* × *str-r1-s3*.

These results become more interesting when considered with the observation of BRYAN (1961) that markers *str-r2* and *str-r3* are nonallelic; they exhibit linkage, being located at different sites on the same transforming molecule. When cells *str-r2* were treated with TP *str-r3*, a recombinant class (*str-r2-r3*) was obtained which had a phenotype distinct from that of *str-r2* or *str-r3* alone; namely, it was capable of resisting up to 4000 μg/ml streptomycin. If the sensi-

TABLE 2

Transformation of sensitive SIII-1 strain by TPs derived from transformants obtained from different allelism tests

TP	Frequency of transformants ($\times 10^{-4}$) resisting: Erythromycin (μg/ml) 0.25	50	Streptomycin (μg/ml) 100	500	1000
A. *str-r2*(TP *str-r1*) *ery-r2* isolate no.					
1	5.6	–*	9.1	8.0	7.4
2	2.5	–	3.7	3.5	3.2
3	4.8	–	1.3	–	1.2
4	1.2	–	2.2	2.3	0.8
5	1.4	–	0.91	1.3	1.5
B. *str-r3*(TP *str-r1*) *ery-r2* isolate no.					
1	2.1	5.1	–	4.4	3.8
2	0.81	0.33	–	0.37	0.35
3	0.65	0.21	–	–	0.19
4	11.0	9.6	–	10.0	8.4
5	0.81	0.62	–	–	0.45
C. *str-r2*(TP *str-r25*) *ery-r2*	2.6	–	3.0	3.4	3.2
	2.2	–	2.6	2.7	2.2
	2.4	–	4.2	3.9	3.4
D. *str-r3*(TP *str-r25*) *ery-r2*	0.18	0.09	–	0.09	0.06
E. *str-r2*(TP *str-r26*) *ery-s2*	–	–	16.5	21.8	18.9
F. *str-r3*(TP *str-r26*) *ery-r2*	0.14	0.17	–	0.10	0.10
G. *str-r2*(TP *str-r29*) *ery-r2*	6.2	0.34	–	0.3	0.3
H. *str-r3*(TP *str-r29*) *ery-s2*	–	0.12	–	0.13	0.14

* Hyphen indicates not tested on this concentration.

Nomenclature of TPs indicates the origin of the strain from which each TP was prepared. For example, in A, the strain was obtained by exposing cells *str-r2* to TP *str-r1*. The *ery-r2* marker was then added to the transformant thus obtained, and a TP prepared. Similar notation used for TPs B-H.

All plates containing 50 μg/ml or 100 μg/ml streptomycin were replicated to plates containing from 1000 to 5000 μg streptomycin, and all colonies were observed to be capable of growth at the higher concentration.

tive strain is treated with the DNA from a transformant of the type *str-r2-r3*, it is found that for every transformant of the double type (*str-r2-r3*) there are 40 of the single type (*str-r2* and *str-r3*).

The results suggest, therefore, that the *str-r1* marker represents a multisite mutation, overlapping the sites of the *str-r2* and *str-r3* markers. It must be strongly emphasized that on the basis of these three experiments the possibility cannot be ruled out that *str-r1* lies between the sites of the *str-r2* and *str-r3* markers with the ends of the *str-r1* marker lying close to those of the adjoining two. These alternatives are shown in Figure 1.

A final analysis of the size of the *str-r1* marker requires the mapping of other markers with relation to the *str-r2* and *str-r3* markers. Evidence that the *str-r1* marker could replace a third marker known to be at a site different from those of the *str-r2* and *str-r3* markers would establish that the *str-r1* marker is, indeed, a multisite mutation. Until such evidence is obtained, the conclusion that marker *str-r1* covers at least two sites must be regarded as tentative. However, if marker *str-r1* does lie between the markers *str-r2* and *str-r3*, it must be of sufficient size to

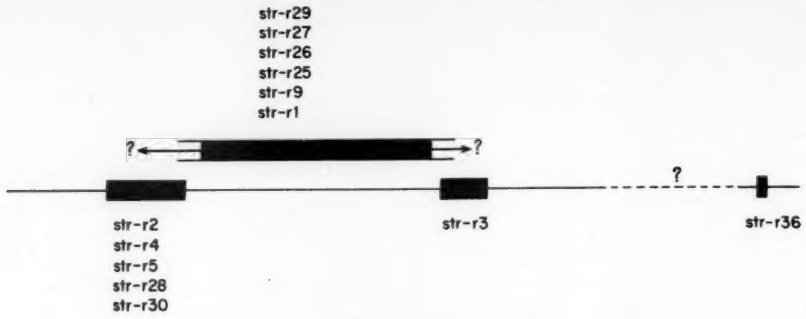

FIGURE 1

MAP OF THE STREPTOMYCIN-RESISTANCE LOCUS

FIGURE 1.—The location of *str-r30* is based on SCHAEFFER (1956). Two possibilities are envisaged for the *str-r1* site. (1) Its ends lie within the segment between the *str-r2* and *str-r3* sites, or (2) its ends overlap one or both of the other sites.

account for the observation that, although *str-r1* must be closely linked to *str-r2* and *str-r3* (since it generally replaces the latter two markers in transformation), markers *str-r2* and *str-r3* exhibit relatively loose linkage.

These data have established at least three sites at which streptomycin-resistance mutations can occur on a single transforming DNA molecule. This suggests that perhaps all streptomycin-resistance markers occur in one region of the bacterial genome and will exhibit linkage to the three sites established above. The following two sections describe the mapping of a random sample of spontaneous mutations to streptomycin resistance.

Alleles of the str-r1 *marker:* The following long-term experiments ("crosses") were performed:

	Cells	TP
1.	*str-r1 ery-s2*	*str-r26 ery-r2*
2.	*str-r1 ery-s2*	*str-r25 ery-r2*
3.	*str-r9 ery-s2*	*str-r1 ery-r2*
4.	*str-r29 ery-s2*	*str-r1 ery-r2*

The two mutations of the last "cross" individually confer approximately the same level of resistance; consequently, transformants were screened for a phenotypic class whose level of resistance would be greater than the level conferred by *str-r1* (>6000 μg/ml streptomycin). Such a class might represent the doubly-marked recombinant type *str-r29-r1*. No phenotypic class of this type could be observed ($<1 \times 10^{-8}$) although the frequency of *ery-r2* transformations (3.5×10^{-3}) indicated that the recipient cells were physiologically competent. Similarly, in the third "cross" no phenotypic class of transformants could be observed with a level of resistance greater than that of *str-r1* ($<1 \times 10^{-8}$) although the frequency of *ery-r2* transformations was 1.6×10^{-4}. In the first two "crosses" trans-

formant classes having the level of resistance of cells containing the markers *str-r26* and *str-r25*, respectively, were obtained. No class having a greater phenotypic level of resistance than that conferred by either *str-r26* or *str-r25* alone was encountered ($<1 \times 10^{-8}$).

Although the phenotypic levels of resistance conferred by the individual markers used in the experiments cited above are not identical, the differences between them are not, in general, great enough to allow a clear phenotypic differentiation between the markers with the methods commonly employed. As has been pointed out previously, given the physiological variation in resistance known to occur in a culture of any mutant strain, complicated by the similarity of the maximum level of resistance of the average cells in the two cultures, identification of the two markers by phenotypic means becomes impracticable. Therefore, should a randomly isolated transformant from any of the experiments described above be genetically a doubly-marked strain, e.g. *str-r9-r1*, a TP prepared from this strain would be of no value since the transformant classes could not be phenotypically distinguished.

Additional evidence for the allelism of these markers is found in the observation that at least three others tested (*str-r25*, *str-r26* and *str-r29*) bear the same relationship to markers *str-r2* and *str-r3*. When strains containing either *str-r2* or *str-r3* were individually treated with either TP *str-r25*, TP *str-r26* or TP *str-r29*, only one class of transformants could be obtained (as determined by direct plating and velvet-replica technique), this class being indistinguishable from cells containing *str-r25*, *str-r26* or *str-r29* alone. When DNA preparations were made from randomly isolated transformants and then tested on the sensitive recipient strain, it was found that genetically as well, only one class was present, this class being identical to that found when the sensitive strain is treated with DNA containing either the marker *str-r26*, *str-r25* or *str-r29* alone (Table 2 C-H). Just as in the case of the *str-r1* marker, there was no evidence of markers *str-r2* or *str-r3* being present simultaneously with either markers *str-r25*, or *str-r26* or *str-r29*.

The simplest explanation is that all these markers (*str-r25*, *str-r26* and *str-r29*) represent different spontaneous mutations at the *str-r1* site. However, it must be pointed out that none of the experimental criteria used offers absolute proof that this is indeed the case. Conclusive evidence must await the mapping of these markers with respect to additional sites of mutation.

BRYAN (1961) has shown by similar analysis that a fifth marker, *str-r27*, occurs at the same site as that of the *str-r1* marker. It is of great interest to note that all markers which apparently fall at this site confer a greater level of resistance than is conferred by markers at the other two sites (*str-r2* and *str-r3*). Furthermore, the double recombinant type *str-r2-r3* has a level of resistance (ca. 4000 μg/ml) almost equal to the level of resistance of *str-r1* (6000 μg/ml). If *str-r1* can be shown unequivocally to be a multisite mutation, then there is a suggestive correlation between the length of the mutation and the level of resistance conferred.

Alleles of str-r2: Cells *str-r5 ery-s2* and *str-r28 ery-s2* were individually

treated with TP *str-r2 ery-r2* (Table 1 C, D). It was observed that if the recombinant classes *str-r2-r5* and *str-r28-r2* exist, they do not have a phenotype distinguishable from marker *str-r2* alone. It was therefore concluded that marker *str-r2* replaces both *str-r5* and *str-r28*, and that the three markers represent different spontaneous mutations at the same site. A fourth marker (*str-r4*) has been similarly shown (BRYAN 1961) to occur at this site. Three of the four markers have identical phenotypes (the exception is *str-r28*), although all confer a much lower level of resistance as compared to *str-r1* and its alleles.

All the markers at this site (*str-r2*, *str-r5*, *str-r28* and *str-r4*) interact with TP *str-r3* to produce the recombinant classes *str-r2-r3*, *str-r5-r3*, *str-r28-r3* and *str-r4-r3* respectively. Furthermore, all markers show the same order of magnitude of linkage to the *str-r3* marker. When the sensitive strain is treated with a DNA prepared from one of these recombinant types, there is about one doubly-marked transformant obtained (e.g. *str-r28-r3*) for every 40 singly-marked transformant (*str-r28* or *str-r3*). Since it has been observed that the degree of linkage (frequency of the doubly-marked class obtained when the sensitive strain is treated with the doubly-marked TP) varies in different experiments with a given DNA preparation,[4] it is not possible to make an absolute comparison of the linkage between *str-r3* and each of the markers cited. However, it is observed that the variations found in the degree of linkage between *str-r3* and each of the markers is no greater than the variation found in repeated tests of one preparation (e.g. *str-r3-r28*).

In each case cited above the recombinant class *str-r3 r*X (where X represents any one of the markers cited) shows a level of resistance far greater than the level of resistance conferred by either of the individual markers. However, the level of resistance of the doubly-marked recombinant classes is not the same in all cases. The combinations *str-r2-r3*, *str-r5-r3* and *str-r4-r3* confer a tolerance of up to 3–4000 μg/ml streptomycin. On the other hand, the combination *str-r28-r3* confers resistance to only about 500 μg streptomycin per ml. This observation coupled with the slightly different level of resistance conferred by marker *str-r28* alone (as compared to *str-r2*, *-r5* or *-r4*) suggested that marker *str-r28* represents a different mutation at the same site.

BRYAN (1961) has shown that an unlinked modifier can increase tenfold the level of resistance conferred by the *str-r2* marker, although this modifier does not alone confer any resistance to the cell. She has also shown that this enhancer (*en*) can increase the resistance conferred by markers *str-r4*, *str-r27* and *str-r1*. It seemed of interest then to determine whether the enhancer would affect all the alleles at this site. Cells *str-r28* and *str-r5* were treated with TP *en str-r2 ery-r2* and it was observed that the enhancer did increase the resistance of *str-r5* cells but could not modify the level of resistance of the *str-r28* cells (Table 3). Since it was possible that the *en str-r28* combination did not result in as high a level of resistance as the *en str-r2* combination, transformants were selected by

[4] Values of 1/20–1/60 have been obtained for the ratio of doubly-marked/singly-marked transformants, in different experiments with the same DNA preparation.

TABLE 3

Transformation of different str-r *markers by TP* en str-r2 ery-r2

Strain	Frequency of cells capable of resisting:* In presence of TP erythromycin (0.25 μg/ml)	In presence of TP streptomycin (1000 μg/ml)	In absence of TP erythromycin (0.25 μg/ml)	In absence of TP streptomycin (1000 μg/ml)
str-r5 ery-s2	2.5	1.58	$< 1 \times 10^{-9}$	1×10^{-9}
	1.0	1.0	..	5×10^{-8}
str-r2 ery-s2	1.0	1.5	..	1×10^{-7}
	1.0	0.9	..	6×10^{-6}
str-r28 ery-s2	6.15	$< 1 \times 10^{-9}$	..	8×10^{-7}
	1.3	3×10^{-7}	..	7.7×10^{-7}
	2.4	1×10^{-7} †	..	4.6×10^{-7}
	4.2	1×10^{-7} †	..	3.0×10^{-7}
			†	1.8×10^{-7}
				4.3×10^{-7}

* $\times 10^{-4}$ unless otherwise indicated.
† Same experiment—replicate tubes.

direct plating on 300 μg/ml, 500 μg/ml and 1000 μg/ml. The results at these three concentrations are identical to those represented for 1000 μg/ml in Table 3. Since the previous data presented indicate that *str-r28* occupies the same site as that of *str-r2*, *-r4* and *-r5*, then it follows that the enhancer is allele specific. That is to say, it does not indiscriminately modify all the alleles at a given site. Moreover, this observation supports the hypothesis that marker *str-r28* represents a different mutation at the same site as that of the *str-r2* marker. Differentiation by means of the enhancer can then serve as a valuable tool for differentiation between the alleles of this locus.

The site of the str-r36 *mutation:* The nitrous acid-induced mutation, *str-r36*, confers a relatively low level of resistance to streptomycin. At concentrations of streptomycin above 35 μg/ml, a significant fraction of *str-r36* mutants are incapable of forming colonies. This compares with a streptomycin concentration of 5 μg/ml, above which a significant fraction of sensitive wild-type (*str-s*X) cells fail to produce colonies. Transformation of the sensitive strain by TP containing the *str-r36* marker can be detected only when the competence of the recipient strain is high, so that the transformants can be distinguished from a background of spontaneous mutants arising from the feeble growth of an appreciable minority of sensitive cells plated at streptomycin concentrations from 20 to 30 μg/ml.

In addition to being able to demonstrate the transfer of the *str-r36* marker into the sensitive clone 3 strain, it has been possible to raise the level of resistance of a clone 3 strain already bearing the *str-r36* marker by transforming it with TP containing either the *str-r3*, *str-r2* or *str-r1* marker. The transformant classes thus obtained are capable of resisting ca. 1000, ca. 5000 and ca. 20,000 μg/ml streptomycin, respectively. While the latter class has a level of resistance indistinguishable from that conferred to clone 3 by the marker *str-r1* alone, the former two classes possess levels of resistance significantly higher than those con-

ferred respectively by markers *str-r3* (400 μg/ml) and *str-r2* (800 μg/ml) alone. On the basis of these results, it may be presumed that *str-r2* and *str-r3* can recombine with *str-r36* to produce, respectively, doubly-mutant *str-r2-r36* and *str-r3-r36* transformants.

Determination of the genetic constitutions of the transformants enhanced in their resistance in this way was accomplished by extracting DNA from them, and testing the DNA preparations on the sensitive clone 3 strain. The results are recorded in Table 4. Transformants were generally selected at three concentrations of streptomycin, and, in addition, colonies produced at the previous concentrations were isolated and tested for their maximum level of resistance.

The significantly greater number of colonies appearing at 25 μg streptomycin per ml than at higher streptomycin concentrations was presumptive evidence that more than one class of transformant was being induced in the sensitive strain. Further evidence came from the marked difference in size of the colonies appearing at 25 μg streptomycin per ml. Many of the colonies that finally developed were not visible at 24 hours of incubation, but were seen as small colonies at 48 hours. These late-appearing or small colonies generally prove to be incapable of resisting 100 μg/ml or higher concentrations of streptomycin; they

TABLE 4

A. *Treatment of sensitive strain with TPs obtained from* str-r36-r*X recombinant types*

TP‡	Expt. no.	Transformants ($\times 10^{-4}$)* capable of forming colonies in agar containing a streptomycin concentration of 25 μg/ml†	100 μg/ml	500 μg/ml	1000 μg/ml
str-r36 (TP *str-r3*)	1	1.55	0.83	$< 10^{-7}$	–
	2	4.7	1.85	3.0×10^{-6}	–
str-r36 (TP *str-r2*)	1	1.62	0.49	–	3.0×10^{-7}
	3	0.15	0.05	–	–
	4	3.65	0.40	–	$< 10^{-6}$
str-r36 (TP *str-r1*)	2	1.23	–	1.0	–
	4	1.68	–	0.33	–

B. *Recovery of distinct classes of transformants*

TP‡	Expt. no.	Colonies selected at streptomycin conc. (μg/ml) of	Total no. isolates tested§	No. of isolates capable of growing at a max. strep. conc. (μg/ml) of 25	100	1000	5000
str-r36 (TP *str-r3*)	1	25	8	6	2	0	0
		100	4	0	4	0	0
	2	500	7	0	0	7	0
str-r36 (TP *str-r2*)	1	25	8	6	2	0	0
		100	4	0	4	0	0
		1000	5	0	1	0	4
	4	25	12 large	2	10	0	0
			6 small	6	0	0	0
str-r36 (TP *str-r1*)	2	25	21 large	0	0	0	21
			80 small	79	0	0	1

* Unless otherwise indicated.
† In case of 25 μg streptomycin/ml, the figures shown are corrected, where necessary, for the small number of colonies appearing in the control plate (no TP).
‡ Nomenclature of TPs as in Table 2.
§ Unless otherwise indicated, isolates are randomly picked from agar containing the concentration of streptomycin indicated. In two of the experiments shown, a separate selection of large and small colonies was made.

correspond, in other words, to a class bearing the *str-r36* marker. The early-appearing or large colonies, on the other hand, generally correspond to a class possessing the other *str-r* marker in the DNA preparation.

From the results obtained by testing individually-isolated colonies, it is clear that the DNA extracted from a transformant presumably *str-r36-r3* in genotype does induce three distinct classes of recombinants in the sensitive strain; transformants corresponding to *str-r36*, *str-r3* and *str-r36-r3*. Similarly, three classes of recombinants, corresponding to *str-r36*, *str-r2* and *str-r36-r2*, are produced in the sensitive strain by DNA extract from the presumed *str-r36-r2* transformant. Finally, two classes of recombinants are produced in the sensitive strain by DNA extracted from the presumed *str-r36-r1* transformant, the two classes corresponding to *str-r36* and to *str-r1*, or *str-r36-r1*, since the phenotype of the latter two types cannot be distinguished. One may conclude therefore that *str-r36* is not allelic with either *str-r2*, *-r3* or *-r1*.

In the case of DNA *str-r36-r3* and DNA *str-r36-r2*, the production in the sensitive strain (*str-s36-s*X) of the doubly-transformed type (*str-r36-r*X) is a relatively rare event. It has never occurred with a frequency greater than one per 100 of the single type (*str-r36* + *str-r*X). This fact, and also the finding that the relative frequency of the doubly-transformed type has varied somewhat with different batches of recipient cells, suggests that the genetic integration of *str-r36* occurs independently of that of either *str-r3* or *str-r2*. In any case, it is evident that *str-r36* is certainly not as strongly linked to *str-r3* and *str-r2* as the latter two markers are to each other.

DISCUSSION

This analysis of 11 randomly isolated, spontaneous mutations has clearly demonstrated that the sites of spontaneous mutation are not randomly distributed in the DNA of pneumococcus. The results of the allelism tests can be summarized by a genetic map (Figure 1) in which the individual mutations have been represented as solid blocks. In addition, the probable location of a twelfth spontaneous mutation *str-r30*, as described by SCHAEFFER (1956), is included for the sake of completeness. For diagrammatic purposes it has arbitrarily been assumed that a mutation conferring a higher level of resistance to streptomycin is larger than one conferring less resistance.

Examination of the map leads to several conclusions. First, it is immediately obvious that there are more mutations than sites. Second, the distribution among the sites can be observed to follow a pattern. All the high level mutations (conferring resistance to 2000 μg/ml streptomycin or more) are found at one site (the site of the *str-r1* marker). At a second site (the site of the *str-r2* marker) there is a group the majority of which confer resistance to 300 μg/ml streptomycin, while the single spontaneous mutation found at the third site confers resistance to only 150 μg/ml. A fourth site, occupied by the only induced mutation examined, confers resistance to only 30 μg/ml streptomycin per ml. The distribution of 12 spontaneous mutations among only three sites begins to suggest a similarity to the finding of frequently mutable sites in bacteriophage

(BENZER 1955) and Salmonella (HARTMAN, LOPER and SERMAN 1960) but which have not previously been reported in pneumococcus. A series of such nonrecombinable (allelic) mutations having similar effects on the phenotype have been designated as a set (HARTMAN *et al.* 1960).

The exact size of the *str-r1* marker and its alleles has not been determined by these studies. It has been shown that there are two main possibilities. Either the ends of these markers overlap the other two sites, or else the ends of these markers lie very close to the other two sites. It is obvious that the two ends of any given marker can be any combination of these possibilities. Since there are only a limited number of linked sites available for mapping purposes, the exact size cannot be determined at the present time. Similarly, it cannot be ascertained if all the members of the set at the *str-r1* site are really identical in length. The absence of recombination between them is merely indicative of the fact they have in common certain regions; in reality, they might represent a series of overlapping multisite mutations of the type observed by LACKS and HOTCHKISS (1960). Preliminary evidence (ROTHEIM, unpublished) suggests that this is so; marker *str-r9* appears to be smaller than the other members of the set. Since this is the marker conferring the lowest level of resistance (among the members of this set), this again seems to confirm the observation that there is a correlation between the level of resistance conferred and the size of the mutation.

Because of the lack of information available concerning the relation of these markers to the actual steps in achieving resistance, it is impossible to talk in terms of functional units. However, the spontaneous mutations can be considered to be part of the streptomycin-resistance locus. This grouping of phenotypically similar mutations into one locus is an observation that has been made repeatedly in many other genetic systems (Salmonella, DEMEREC 1956; HARTMAN *et al.* 1960; bacteriophage T4, BENZER 1957; Neurospora, CASE and GILES 1960). Likewise, the differences observed between different mutations at this locus (differences in the level of resistance) parallel the secondary differences observed between mutations affecting the same function in Salmonella (YURA 1956; CLOWES, cited in DEMEREC 1956), bacteriophage (BENZER 1955; STREISINGER, MARTINELLO and WASSERMAN 1957), and Neurospora (GILES 1959; DE SERRES 1958). The primary function we can assign to the mutations in the case reported here is streptomycin resistance. Finally, there is a differentiation observed between the members of a set. There are five markers located at the site of the *str-r2* marker. Three of these (*str-r2*, *-r4* and *-r5*) confer approximately the same level of resistance (300 μg/ml streptomycin) and in combination with *str-r3* all produce a double recombinant type (e.g. *str-r2-r3*) which has a level of resistance of about 3–4000 μg/ml streptomycin. Furthermore, these three markers are all affected by an enhancer gene (BRYAN 1961) which increases the level of resistance tenfold. Marker *str-r30*, which we have not studied, appears to differ in at least one respect: it confers a very low level of resistance (25 μg/ml). Marker *str-r28*, however, differs in all three respects. First, it has a lower level of resistance. Second, the combination *str-r28-r3* gives a much lower level of resistance (ca. 500 μg streptomycin per ml). Third, the

enhancer gene has no effect on marker *str-r28*. These findings indicate that even at one site different mutational changes can occur, and the enhancer mutation can provide a useful tool for differentiating between the members of a locus. Indeed, BRYAN (1961) has shown that the enhancer affects at least two markers at the *str-r1* site (*str-r1* and *str-r27*). It is interesting to note that, should *str-r1* prove to be a multisite mutation, the specificity of the enhancer is such as not to affect all the markers "covered" by the multisite mutation while affecting the multisite mutation itself.

The site of the nitrous acid-induced mutation, *str-r36*, is apparently distant from the three sites of spontaneous mutation that have been discovered. It would be premature to conclude, however, a specificity in the action of this mutagen. An insufficient number of spontaneous mutations selected at low streptomycin concentrations have been analyzed to be sure that the sites of spontaneous mutation are separate from the sites of induced mutation. Only two facts bearing on this question are available. First, in our own experiments, as well as in those of SCHAEFFER (1956), we have found spontaneous mutations that confer resistance to only 20–30 μg streptomycin per ml. Secondly, the only spontaneous mutation of this type to have been studied, *str-r30*, was found by SCHAEFFER to be allelic with *str-r28* and *str-r29*, which we in turn have found to be members of the locus just described (Figure 1). While suggesting that spontaneous mutations conferring low levels of resistance occur within the same locus as spontaneous mutations conferring high levels of resistance, the evidence is insufficient to generalize.

While it is possible to state that the site of *str-r36* is not strongly linked to the sites of spontaneous mutation that have been described, it will require further investigation to determine whether the site of the induced mutation is transferred independently of the sites of spontaneous mutation during pneumococcal transformation. It is even possible that two mutations transferred independently of each other (i.e. *genetically* "unlinked") may be borne on the same molecule of DNA (i.e. *physically* "linked"). Physical and genetic experiments to elucidate this point are now being attempted.

SUMMARY

1. Eleven independently-derived spontaneous mutations to streptomycin resistance in Pneumococcus were found to be distributed among three linked sites of a transforming DNA molecule.

2. The distribution was observed to be non random in that there is a definite tendency for mutations conferring similar resistance to be found at the same site.

3. At any one site, more than one type of mutation has been shown to occur, and it has been possible to differentiate between the mutations by means of a modifier gene.

4. The site of a mutation induced by nitrous acid, which confers only a very low level of resistance to streptomycin, is not strongly linked to the sites of the spontaneous mutations.

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ON THE MECHANISM OF ABNORMAL TRANSMISSION RATIOS AT T LOCUS IN THE HOUSE MOUSE[1]

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Received August 4, 1961

IT has generally been held that the behavior of sperm in effecting fertilization is not affected by the genes which they transmit. One basis for this assumption is that gametes from heterozygous animals usually unite at random in fertilization since this is one condition for the production of normal Mendelian ratios. In cases in which abnormal ratios are regularly obtained from heterozygotes, this assumption is brought into question and a causal analysis is required.

Genetical evidence of abnormal transmission ratios of alleles at one locus in the house mouse was disclosed many years ago (CHESLEY and DUNN 1936) and has been reviewed recently (DUNN 1960). The pertinent facts for present purposes are that whereas females hetrozygous for any one of a series of alleles, known collectively as *t* alleles, transmit both alleles in equal proportions, males heterozygous for the same pairs transmit them in proportions that under normal conditions are markedly unequal. Usually males heterozygous for a lethal *t* allele transmit it to from 70–99 percent of their progeny, depending on the *t* allele concerned. These are known as "high-ratio *t* alleles." In a few cases "low-ratio" lethal *t* alleles having transmission ratios below 50 percent have been found, as well as *t* alleles which are viable when homozygous, different ones of which are transmitted by males in high, low or normal ratios. The present work deals only with high-ratio lethal alleles.

Until 1958, no theory of the mechanism responsible for high male transmission ratios had been supported by experimental evidence. Then BRADEN (1958) found that males heterozygous for certain *t* alleles transmitted them in high ratios when matings with test females occurred at the normal time i.e. coitus about five hours before ovulation; whereas the same males, when permitted to mate only after ovulation in the test females had recently occurred (late matings), usually transmitted the *t* alleles in ratios significantly lower than after normal matings. In some cases the ratios obtained after late mating were close to the normal 50 percent. The latter result suggested that sperm carrying such *t* alleles were produced in normal proportions; and BRADEN proposed an hypothesis that the effects of each of three high-ratio alleles (t^0, t^1, t^{12}) were exerted on the sperm which carried such an allele in the interval between ejaculation and fertilization. The probability of success of such sperm in achieving fertilization was assumed to be

[1] Research carried out at the Department of Zoology and at Nevis Biological Station, Irvington-on-Hudson, N.Y. under contract AT (30–1) 1804 with the U.S. Atomic Energy Commission.

increased when the interval between coitus and fertilization was longer (as in normal mating) but decreased toward normal proportions when this interval was shortened (as in late matings).

Of seven males heterozygous for high-ratio *t* alleles, as tested by BRADEN, six responded to late mating by showing significant decreases in the proportion of offspring receiving *t*, while the ratio from one did not change. One male heterozygous for a viable allele (t^3) showed a normal transmission ratio after normal mating but a significantly lower ratio of *t* after late mating.

These results supported BRADEN's general hypothesis that the mechanism responsible for the abnormal ratios has a physiological basis in effects of *t* alleles on sperm behavior rather than a cytological one in effects on meiosis. It raised new questions concerning the extent to which the general hypothesis could be applied to *t* alleles with very high transmission ratios, such as those found in wild populations (DUNN 1960), and to those with abnormally low ratios, and other questions concerning the nature of the mechanism.

The experiments reported in this paper were designed to test the hypothesis first as applied to one of the *t* alleles studied by BRADEN which had a moderately high ratio, with special attention to variations amongst males of the same *t* genotype, and second as applied to *t* alleles with very high ratio. Tests of low-ratio alleles and of more specific hypotheses concerning the mechanism are now under study and will be reported later.

Methods used for controlling ovulation time

Our methods were essentially similar to those used by BRADEN based on the relation between the diurnal light cycle and the time of ovulation (BRADEN 1957). We carried out preliminary experiments to test this relation in the stock which was to furnish the normal test females to be used in the late mating experiments. This stock, different from that used by BRADEN, was ICR albino (normal tailed) as maintained by Camm Research Institute Inc., Wayne, N.J. Young adult (4–6 weeks old) virgin females were supplied to us as required.

When males and females are kept together mating usually occurs about five to eight hours before ovulation; that is, those females which are about to ovulate accept the male at this time. To test control of ovulation time we kept groups of ICR females for three weeks under a cycle, controlled by a clock switch, of ten hours light (9 AM to 7 PM) and 14 hours dark (7 PM to 9 AM). A fertile male was then added for a short period to each pen of five females. Different pens received a male from 1–2 AM, 4–5 AM, 7–8 AM, 8–9 AM, 9–10 AM, 11 AM–1 PM, 1–3 PM, 3–5 PM, 5–7 PM, 11 PM to midnight and 12–1 AM. Females were subsequently examined during the dark period under red light for vaginal plugs to identify those that had mated in the specified period. Females which had mated were dissected, some at one, two, four or six hours after mating and the Fallopian tubes were examined for the presence of eggs. Verification of mating was made by finding sperm in the female reproductive tract.

Table 1 and Figure 1 show the results of examination for eggs. Mice of this

TABLE 1

The increase in the number of albino mice ovulated at each time under the light cycle of ten hour light and 14 hour darkness

Time of day	No. of mice examined	No. of mice with eggs in oviducts	Percentage of mice that had eggs in oviducts
12:00 Midnight	5	0	0
2:00 AM	5	0	0
4:00 AM	7	1.5*	21.4
6:00 AM	6	5.0	83.3
8:00 AM	6	4.5	75.0
10:00 AM	6	5.5	91.7
11:00 AM	6	5.5	91.7
12:00 Noon	6	6.0	100.0
2:00 PM	6	6.0	100.0
4:00 PM	2	2.0	100.0
6:00 PM	2	2.0	100.0
8:00 PM	6	6.0	100.0

* 1.5 . . . This means one female ovulated eggs from both the ovaries and one female ovulated eggs from one of the ovaries.

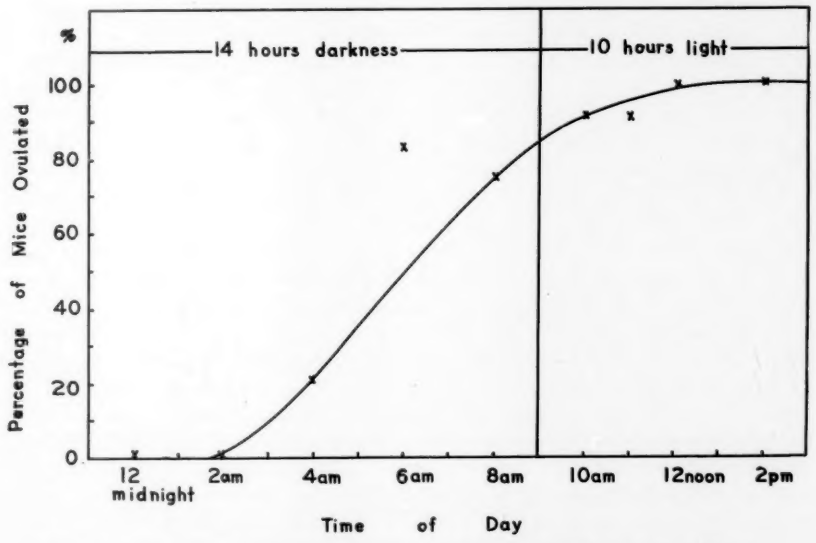

FIGURE 1.—Showing the progressive increase in the percentage of the mice ovulated.

stock under this light cycle begin to ovulate about 3 AM and by noon all have ovulated. Ovulation does not always occur at the same time from both ovaries. In seven out of 63 females dissected ovulation had occurred in only one ovary. The progress of ovulation under a controlled light cycle was thus similar in our

stock and in those studied by BRADEN (1957) although he had found small differences between stocks.

In devising a routine system of control for our late mating experiments we took advantage of BRADEN's (1957) finding that there was less variation among females kept under a light cycle of four hours of dark and 20 hours of light than under a longer dark period. The former also required less time for the completion of ovulation. The midpoint of the ovulation period appears to be determined largely by the midpoint rather than the extent of the dark period. For our tests of the effect of late mating on the male transmission ratio we adopted the regimen of keeping test females for 2–3 weeks under a cycle regulated by a clock-switch of four hours dark (midnight to 4 AM) and 20 hours light. The midpoint of the dark period (2 AM) was thus the same as in the ten hour dark period previously tested. Under such conditions females should complete ovulation by noon.

Methods of testing transmission ratios

Males to be tested were tailless of genotypes T/t^1, T/t^{w6} and T/t^{w10}, the first being one of the genotypes tested by BRADEN, the latter two containing lethal t alleles derived from wild populations. When mated with normal $(+/+)$ females, such males produce two classes of offspring phenotypically distinct at birth: those with normal tails $(+/t)$ and those with short (Brachy) tails $(+/T)$. The ratio between these provides an estimate of the relative frequency of sperm with t and with T which effect fertilization. The relative viability of the two phenotypes to birth is about equal as shown by the equal frequency of the two types at birth from female heterozygotes $(T/t\,♀ \times +/+\,♂)$ and from males with t alleles having normal transmission ratios.

The ratios from tailless (T/t^n) males were tested first under normal mating and normal light conditions. Each male was penned continuously with five normal females in a room which was light in the day time and dark at night. Females were removed when pregnant and bore their litters in isolation. After scoring for tail length within 24 ± 12 hours after birth, the young were killed and the mother was mated again, after an interval of 1–2 days with a different male. After a number of such litters had been scored, each male was then tested by females which had been kept under the light cycle of four hours dark and 20 hours light. These comparisons produced the results shown in Table 2. It is evident that matings under the two sets of conditions produced transmission ratios which are in general similar. Deviations from the average ratios occurred in both directions and there was significant and similar heterogeneity amongst such males under both sets of conditions. In the case of one male, the ratio obtained under artificial light departed widely and significantly from the ratio obtained under normal light and this contributed 20 to a total χ^2 of 56 for matings under artificial light. This was clearly exceptional. The causes of heterogeneity will be discussed later but we concluded that alteration of the light cycle was not an important cause of major change in the transmission ratios.

TABLE 2

The male transmission ratio of alleles t *and* T *after normal mating under the normal light condition and the artificial light condition. (4 hrs. darkness, 20 hrs. light)*

Genotype of male	Normal light condition			Artificial light condition			Significance of difference
	No. of offspring			No. of offspring			
	t/+	*T*/+	Ratio	*t*/+	*T*/+	Ratio	
t^1/T							
I	57	20	74.0:26.0	47	16	74.6:25.4	N. S.
II	35	10	77.8:22.2	59	11	84.3:15.7	N. S.
III	34	5	87.2:12.8	28	27	50.9:49.1	$P < 0.005$
IV	23	5	82.1:17.9	82	47	63.6:36.4	$P < 0.005$
V	61	17	78.2:21.8	70	15	82.4:17.6	N. S.
VI	51	3	94.4: 5.6	55	5	91.7: 8.3	N. S.
VII	89	23	79.5:20.5	39	18	68.4:31.6	N. S.
VIII	66	6	91.7: 8.3	79	9	89.8:10.2	N. S.
IX	40	17	70.2:29.8	68	13	84.0:16.0	N. S.
X	59	16	78.7:21.3	34	16	68.0:32.0	N. S.
XI	70	15	82.4:17.6	52	17	88.1:11.9	N. S.
Total	585	137	81.0:19.0	613	184	76.9:23.1	$P < 0.01$
t^{w6}/T							
III	39	1	97.5: 2.5	80	0	100.0: 0.0	N. S.
IV	75	0	100.0: 0.0	68	0	100.0: 0.0	N. S.
Total	114	1	99.1: 0.9	148	0	100.0: 0.0	N. S.
t^{w10}/T							
I	26	0	100.0: 0.0	91	5	94.8: 5.2	N. S.
II	66	3	95.6: 4.4	51	11	82.3:17.7	$P < 0.005$
VI	32	5	86.5:13.5	31	2	93.9: 6.1	N. S.
VII	64	4	94.1: 5.9	66	7	90.4: 9.6	N. S.
Total	188	12	94.0: 6.0	239	25	90.5: 9.5	N. S.

Effects of late mating

The males tested as above were then tested in parallel under artificial light conditions for ratios after normal mating and after late mating. Each male, penned continuously with five normal females for normal mating, was removed from 1–3 PM to another pen of five females under artificial light cycle. Matings which occurred in this period would thus take place after ovulation had occurred, at an average interval of about two hours. Each female found pregnant was removed to bear her litter in isolation. After the litter was scored and killed, the mother was added to a pool of unmated females to be kept there for two weeks before being mated with a different male. This was to permit resumption of normal oestrus and to randomize the females mated to each male.

The results of these tests are shown in Tables 3 and 4. In Table 3 are the ratios obtained from tailless males, T/t^1. The ratios from late matings are generally lower than those from normal matings and significantly so in all except tests of three males. One of these (III) which had given a normal ratio under normal

mating gave a significantly higher ratio under late mating, i.e. the change in this ratio is in the direction opposite to that which occurred in other males. The average reduction in ratio from 77 to 64 percent is highly significant and in the same direction as results of BRADEN's (1958) tests of males with the same *t* allele. Our results with this allele thus confirm BRADEN's conclusions.

In Table 4 are the ratios obtained from tailless males heterozygous for two high-ratio *t* alleles derived from wild populations. There are, with one exception,

TABLE 3

The male transmission ratio of the alleles t *and* T *after late mating and after normal mating under the normal light condition*

Genotype t^i/T male	Normal mating No. of offspring $t/+$	$T/+$	Ratio	Late mating No. of offspring $t/+$	$T/+$	Ratio	Significance of difference
I	47	16	74.6:25.4	32	19	62.7:37.3	$P < 0.025$
II	59	11	84.3:15.7	26	9	74.3:25.7	N. S.
III	28	27	50.9:49.1	50	25	66.7:33.3	$P < 0.01$*
IV	82	47	63.6:34.4	77	44	63.6:36.4	N. S.
V	70	15	82.4:17.6	24	16	60.0:40.0	$P < 0.005$
VI	55	5	91.7: 8.3	102	39	72.3:27.7	$P < 0.005$
VII	39	18	68.4:31.6	54	29	65.1:34.9	N. S.
VIII	79	9	89.8:10.2	50	40	55.6:44.4	$P < 0.005$
IX	68	13	83.9:16.1	24	14	63.2:36.8	$P < 0.005$
X	34	16	68.0:32.0	45	36	55.5:44.5	$P < 0.005$
XI	52	7	88.1:11.9	15	15	50.0:50.0	$P < 0.005$
Total	613	184	76.9:23.1	499	286	63.6:36.4	$P < 0.005$

* Ratio of t^i *increased*.

TABLE 4

The male transmission ratio of the alleles t *and* T *after late mating and after normal mating under the normal light condition*

Genotype of male	Normal mating No. of offspring $t/+$	$T/+$	Ratio	Late mating No. of offspring $t/+$	$T/+$	Ratio	Significance of difference
t^{w6}/T							
I	64	5	92.8: 7.2	99	8	92.5: 7.5	N. S.
II	90	1	98.9: 1.1	76	0	100.0: 0.0	N. S.
III	39	1	97.5: 2.5	54	1	98.2: 1.8	N. S.
IV	75	0	100.0: 0.0	54	0	100.0: 0.0	N. S.
Total	268	7	97.5: 2.5	283	9	96.9: 3.1	N. S.
t^{w10}/T							
I	26	0	100.0: 0.0	84	5	94.4: 5.6	N. S.
II	66	3	95.7: 4.3	30	0	100.0: 0.0	N. S.
III	97	5	95.1: 4.9	96	6	94.1: 5.9	N. S.
IV	98	6	94.2: 5.8	96	6	94.1: 5.9	N. S.
V	48	1	98.0: 2.0	30	10	75.0:25.0	$P < 0.005$
Total	335	15	95.7: 4.3	336	27	92.6: 7.4	N. S.

no significant differences between ratios from normal and from late matings and it is clear that the average results are the same for normal and late matings.

DISCUSSION

Taken at face value the above results appear to show first that males transmitting the allele tested by BRADEN respond to our technique for obtaining late mating in much the same way that they did to BRADEN's, thus providing confirmation of his original observation and an indication that our methods and his are comparable. Second, these methods applied to males heterozygous for very high-ratio alleles fail to produce any significant response.

Before attempting to reconcile these results with any hypothesis concerning the mechanism involved, it is necessary to examine the variation in the ratios produced by males of the same t genotype under one set of mating conditions. The ratios are highly variable as judged by those produced by T/t^1 males under normal mating (Table 1). Under normal light, the proportions of offspring receiving t^1 vary from 70.2 to 94.4 percent; under the short dark cycle from 50.9 to 91.7 percent. In each case there is significant heterogeneity amongst the males in this respect as judged by χ^2†. For 11 T/t^1 males under normal light $\chi^2 = 19.899$; d.f. $= 10$; $P = 0.028$. For the same males under the short dark cycle $\chi^2 = 55.706$; d.f. $= 10$; $P < 0.001$. There are probably many causes of this variability (variations in time of mating and of ovulation, in genotype other than at T locus etc.) but one fact of special importance is the non random distribution of frequencies of the two phenotypes appearing in successive litters from the same male. This phenomenon which was called "clustering" when first discovered (DUNN 1943) occurs frequently, perhaps usually, in males with a t allele showing abnormal transmission ratios, but not in those with t alleles showing normal ratios nor in females (DUNN 1960). This "within-male heterogeneity" is evident in some of the T/t^1 males both under normal and under late-mating conditions. For ten litters produced by male X under normal mating $\chi^2 = 24.825$; d.f. $= 9$; $P = 0.003$; for nine litters from this male under late mating $\chi^2 = 21.048$; d.f. $= 8$; $P = 0.007$. This phenomenon is to be analysed later in its relation to hypotheses of the mechanism of the late-mating effect. For present purposes its chief importance is to show that variations in ratios are not normally distributed, and this evidence of non random variation throws doubt on the applicability of binomial probabilities in computing the sampling errors of ratios to be compared under different conditions. Until a theory for this is worked out, the estimates of significance of differences in ratios, as in Table 3, are subject to error from this source. Judgments concerning changes in ratios under late mating should thus be based on the general consistency of the results rather than upon individual estimates of significance. Of the 11 T/t^1 males tested, the t^1 transmis-

† χ^2 is computed as $\Sigma \frac{1}{xy}\left[\frac{(Ny_i - N_i y)^2}{N_i}\right]$ in which x and y are the total number of normal $(+/t^1)$ and of Brachy $(+/T)$ offspring produced by males; N the sum of these, y_i, the number of Brachy and N_i, the total offspring for each male.

sion ratios of nine decreased under late as compared to normal mating, one did not change, and one increased. The tendency is clearly to decrease but the overall quantitative change is small—from 76.9 percent under normal to 63.6 percent under late mating. The changes obtained by BRADEN (1958) for two males of this genotype were greater—from about 76 percent to 53 percent. In BRADEN's tests, two males of a different *t* genotype (T/t^0) also responded to late mating with a smaller change in ratios i.e. from 82 percent to 60 percent; while three males (T/t^{12}) giving the highest ratios under normal mating responded to late mating with the least change of ratio i.e. 91 percent to 78 percent. Finally, in our tests, eight males giving still higher ratios (Table 4) under normal mating did not respond at all to late mating while one showed a decrease.

One conclusion that can be drawn from these comparisons is that the degree of response to late mating depends on the magnitude of the abnormal ratios obtained under normal mating, the highest ratios showing the least change. An interpretation of the late-mating effect must therefore take into account both the lack of uniformity in response of males of the same *t* genotype, and the quantitative differences between the *t* alleles tested in the degree of their response. It should also be remembered that some *t* alleles are transmitted by heterozygous males in normal ratios under normal mating while others are transmitted in low ratios.

We shall assume first that a specific *average* transmission ratio under normal mating is an inherent property of each *t* allele. This may not be strictly true since we do not know the effects of other genotypic variations on the ratios. Second we shall assume that these properties are expressed through the same general type of mechanism for all *t* alleles and, following BRADEN, that effects of this type are exerted by the allelic state of the *T* locus in the sperm after the completion of meiosis. This implies that *t* and non-*t* sperm are produced in equal numbers by heterozygous males. We have no strict proof of this assumption. The proportions of offspring receiving *t* and non-*t* alleles from males transmitting certain *t* alleles in normal ratios under normal mating cannot be used as evidence until it is shown that such ratios are not altered after late mating. The ratio from one such male (T/t^3) tested by BRADEN (1958) was in fact altered *away* from normal by late mating. A single instance may not be typical. If it proves to be so, it will constitute evidence against the hypothesis proposed here. The point is now being tested experimentally. In favor of the assumption that *t* alleles exert their effects after meiosis is first of all the fact that in cases in which abnormal ratios are affected by late mating, the effect is prevailingly toward normal ratios. Secondly there is no evidence of meiotic irregularity to account for the abnormal ratios although this is of minor value since descriptions of meiosis in a variety of males with *t* alleles showing abnormal ratios are not available.

In any case, for purposes of simplicity, we shall assume that at some moment after meiosis, *t* and non-*t* gametes are present in equal numbers in males heterozygous for a *t* allele. Following meiosis, the gametes are subject to storage for variable lengths of time before moving through epidydimis and vas deferens to ejaculation. The ratios of *t* and non-*t* sperm are subject to alteration by reactions

between their genotypes and their environment at any time during this period. If the effects are exerted after ejaculation, as proposed by BRADEN, the ratios of sperm of the two genotypes should be equal when they enter the female reproductive tract and if the interval between this time and the time of fertilization is short, as under late mating, then the results of late mating should tend to express this ratio. But if the effects are exerted earlier, before ejaculation, then the ratios will already have been altered, and the interval between ejaculation and fertilization should have less effect or none at all upon the ratio at fertilization. We shall adopt this as a working hypothesis to explain the differences in response to late mating of males heterozygous for *t* alleles differing in the magnitude of the ratio abnormality under normal mating.

One advantage of this hypothesis is that it is subject to test by direct observations of sperm. It predicts for example that in males with *t* alleles which respond to late mating by regression toward normal ratios, two physiologically different categories of sperm should be found at or near the time of ejaculation in proportions approaching those observed genetically after late mating. In males with *t* alleles which do not respond to late matings, the proportions found genetically should correspond to those observed physiologically near the time of ejaculation. This would mean in the case of very high ratio alleles such as t^{w6} or t^{w10} (Table 4) that nearly all sperm before ejaculation should be of one physiological type, i.e. the behavior expressed by t^{w6} or t^{w10}. Such observations are in progress.

Finally the question can be raised whether a physiological hypothesis of this type can account for the non random distribution of frequencies of fertilization by the *t* and non-*t* sperm in different litters of the same heterozygous male under normal mating. If we assume that this so-called cluster phenomenon is associated with the interval between copulation and ovulation, and that some females, by chance, accept the male near to the time of ovulation, then the frequencies in such litters should resemble those obtained under late mating and show higher than average frequencies of fertilizations by non-*t* sperm. If this is so then "within-male" heterogeneity in ratios in successive litters should be reduced when all matings are artificially restricted to late matings. And if heterogeneity in the ratios produced by males of the same *t* genotype is due to the above cause then "between-male" heterogeneities should also be reduced under late mating. Some computations from our present data on T/t^1 males under normal and under late mating have given no indication that late mating significantly reduces heterogeneity. The χ^2 estimates for the males in Table 1 have already been given. In addition male VI in 14 litters under late mating showed $\chi^2 = 54.474$; d.f. $= 13$; $P = 0.001$, and male VII in nine litters $\chi^2 = 25.512$; d.f. $= 8$; $P = 0.001$. We take this to mean that other factors are involved in clustering in addition to or apart from the interval between copulation and fertilization. Further study of these is required.

SUMMARY

Evidence has been given that males heterozygous for one allele at locus *T* (11 T/t^1 males tested) tend to transmit the t^1 allele to a higher proportion (77 percent) of their offspring when mating occurs at the normal time, than when mating is artificially restricted to a two-hour period closer to the time of ovulation (average ratio after late mating, 63.6 percent). There is however considerable variation in the ratios between males both under normal and late mating and within litters from the same males under both sets of conditions. In general the results obtained are in the same direction as those reported by BRADEN (1958) for this allele.

Of nine males heterozygous for *t* alleles giving very high *t* transmission ratios (T/t^{w6} and T/t^{w10}) under normal mating (average 96 percent) eight showed no change in transmission ratio after late mating, one showed a small decrease in *t* offspring (75 percent).

In accounting for these results a modification of the hypothesis proposed by BRADEN is adopted whereby the varied transmission ratios associated with different *t* alleles in heterozygous males are assumed to be due to physiological effects exerted by a *t* allele on sperm which transmit it at some time between meiosis and fertilization. Alleles whose transmission ratio is lowered by reduction of the interval between copulation and ovulation (as in late mating) are assumed to affect sperm behavior between ejaculation and fertilization; those which retain high transmission ratios after late mating, are assumed to have their effects on sperm before ejaculation. Methods of testing this hypothesis are proposed.

The hypothesis, in this form, does not by itself account for the non random distribution of frequencies of the high-ratio alleles in fertilizations effected under normal mating.

ACKNOWLEDGMENTS

We are grateful to PHEBE VAN VALEN for making preliminary tests of methods of controlling ovulation by hormone treatment and for her help in the early stages of the work reported in this paper.

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GENETIC CONTROL OF NUCLEAR SELECTION IN NEUROSPORA HETEROKARYONS[1]

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Received August 7, 1961

THE lack of stable diploids in Neurospora has made it necessary for geneticists using this organism to rely almost exclusively on heterokaryotic systems in genetic tests requiring heterozygosity. Following the pioneering work of BEADLE and COONRADT in 1944 on heterokaryosis, it has been repeatedly demonstrated that when compatible strains of the appropriate genotypes are employed, heterokaryotic systems can be exteremely useful in the study of a variety of genetic phenomena. However, if valid conclusions are to be drawn from experiments using heterokaryotic systems merely as a tool, the systems themselves must first be thoroughly understood. Although heterokaryons probably are more widely used now than ever before, many purely biological aspects of heterokaryotic growth and behavior are still incompletely understood, partially because of the scarcity of information regarding the interaction of genetically different nuclei in such systems. That the ability of different strains to form a stable heterokaryon is genetically controlled (GARNJOBST 1953, 1955; HOLLOWAY 1955) has been known for some time. Still many genetic factors effecting heterokaryotic compatibility and growth remain undescribed. More important perhaps, the modes of action of such genes, although unknown, are obviously different in some cases from the cytoplasmic incompatibility described by GARNJOBST and WILSON (1956) and WILSON, GARNJOBST and TATUM (1961). If the phenotypes of heterokaryotic cultures are to provide useful criteria for assessing nuclear interactions, more information is needed concerning the extent to which genetic factors directly control the many abnormal phenotypes regularly encountered in the growth of heterokaryotic cultures. Such incompatible phenotypes include not only complete lack of heterokaryotic growth, but also labile growth rates, cyclic patterns of growth and cessation of growth, submaximal growth rates, normal growth followed after a time by complete cessation of growth, or no growth for various lengths of time followed by growth, either maximal or submaximal.

Since some of the same growth phenotypes used to define compatibility and incompatibility are also used in assessing complementation and noncomplementation, it is most important to be able to distinguish between factors effecting

[1] Contribution No. 753, Department of Agronomy, Kansas Agricultural Experiment Station, Manhattan. This investigation was supported by a grant, G9029, from the National Science Foundation.

these two phenomena. For example, in intra-allelic complementation studies it is commonly found that two mutant strains, A and B, may complement, and that A also complements strain C, but B and C do not complement. It is apparently not commonly known, however, that exactly the same situation may exist in regard to compatibility reactions, and moreover, that such reactions can be demonstrated with nonallelic nutritional mutants (HOLLOWAY 1953). Such observations suggest that homozygosity of certain genetic factors is not enough in itself to insure compatibility; interactions between nonallelic genes must also be considered as contributing to incompatibility reactions. Although it is routinely possible to avoid the more obvious complications by striving for homozygosity, it is not completely clear whether such a condition is always desirable. This may be inferred from the observations that many single gene differences arising through mutation may have a more adverse effect when they occur in homozygous strains (EMERSON 1947, 1948; EMERSON and CUSHING 1946; RYAN and LEDERBERG 1946; and DAVIS 1960b), although it remains to be shown whether such mutations would behave similarly in more heterogeneous systems in all cases. In any event it is clear that when heterokaryons are used as a tool in studying genetic phenomena, every precaution must be taken to make certain that the observed phenomena do not result from interactions of genetic factors effecting heterokaryosis itself rather than from the genetic system under investigation.

Although the many problems inherent in studying genes whose only phenotype is recognized in heterokaryons makes such an analysis a formidable one, this study was initiated with the idea of searching for genes whose interaction is manifested as nuclear competition. In studies of genetic factors effecting heterokaryon compatibility the authors have been impressed with the number of strains eventually classified as incompatible which actually grow as heterokaryons for considerable distances in growth tubes before stopping. These observations suggested that such behavior may be due to a nonadaptive increase in one of the nuclear components resulting in homozygosity. To determine more precisely if such mechanisms are commonly operative, methods especially suited for detecting such behavior were developed. This report is concerned with a description of an allelic pair of genes, designated *I* and *i*, which have just such an effect and whose phenotype at present can be detected only in heterokaryons. Nuclei carrying *I* are operationally able to inhibit the normal multiplication of *i* nuclei under specified conditions leading to a nonadaptive increase in *I* nuclei. However, *I* is classified as being weakly dominant since, if the proportion of *i* nuclei is approximately .70 or greater, *I* nuclei are then incapable of affecting the division rate of *i* nuclei and growth is normal.

Theoretical notations and descriptions of the system used to detect nuclear selection in Neurospora heterokaryons: It previously has been demonstrated that the nuclear proportions in heterokaryons among compatible strains carrying different nutritional mutants show no systematic change during prolonged growth under varying environmental conditions. The stabilizing mechanism in such balanced heterokaryons is a nonadaptive one and operates to maintain the status quo even when a change would be advantageous in terms of growth rate of

the culture. The stabilizing mechanism involves an active transport system, plus being consistent with the hypothesis that different nuclei in the same heterokaryotic culture have the same rate of nuclear division (PITTENGER and ATWOOD 1956). DAVIS (1959) confirmed that hypothesis by showing that the nuclear ratios in heterokaryons maintained on minimal agar slants through several conidial transfers stabilized around 50 percent of each nuclear type as would be expected if nuclear division rates were equal and heterokaryotic conidia were the only contributors to each new transfer generation. This does not mean, however, that labile growth rates do not occur, nor that such rates are not correlated with changes in nuclear ratios. Systematic changes in nuclear proportions may and do result from combinations of genotypes that do not fulfill the conditions of equal growth rates. The unstable type III heterokaryon described by HOLLOWAY (1955) is an excellent example, as are the non-adaptive growth reported by RYAN and LEDERBERG (1946) and a similar case reported by DAVIS (1960b).

That different nuclei in a heterokaryon normally divide at the same rate can be demonstrated by using conidial-derived heterokaryons. When conidia from a heterokaryon are plated on minimal sorbose medium, each colony is assumed to have originated from a single heterokaryotic conidium. A culture derived from such a conidium will be referred to as a conidial-derived heterokaryotic culture. It has been demonstrated that heterokaryons grown on minimal medium have an average of between two and three nuclei per conidium (PROUT, HUEBSCHMAN, LEVENE and RYAN 1953; HUEBSCHMAN 1952; ATWOOD and MUKAI 1955). If rates of nuclear division are equal, a first approximation prediction would be that cultures established from heterokaryotic conidia should have nuclear proportions similar to those found in individual heterokaryotic conidia, that is, ratios of approximately 1:1, 2:1, 1:2, and less often 3:1, 1:3, 2:3, 3:2, 4:1 and 1:4. Approximations of such ratios have been found repeatedly in our laboratory in conidial-derived heterokaryotic cultures using a variety of nutritional mutants. In certain nuclear combinations the frequency of the more disparate nuclear ratios is higher than expected. In a few cases these can be accounted for by the presence of mycelial fragments from heterokaryons with disproportionate nuclear ratios, but in most instances a lack of synchronism in the early nuclear divisions within germinating conidia is probably involved. This lack of synchronism of nuclear divisions within conidia can be demonstrated cytologically (C. SOMERS, personal communication) and undoubtedly often leads to some distortion of the expected nuclear ratio in heterokaryons derived from single conidia. For example, if a conidium has two type-A and one type-B nuclei and if both type-A nuclei divide once before the type-B nucleus divides at all, and thereafter all nuclei divide at an equal rate, the ratio in the mature heterokaryon would approximate four A to one B rather than the expected two A to one B. During this period of germination and early growth of the conidium before an equilibrium in cellular processes is reached, many possible mechanisms might distort somewhat the initial nuclear ratio present in individual conidia.

Although the nuclear ratios in conidial-derived heterokaryotic cultures can usually be accounted for by nuclear ratios known to exist in heterokaryotic

conidia, whether the exceptions are stable and have genetic bases has never been systematically determined. Under the usual experimental conditions, however, deviations from the expected ratio are detected only when special techniques involving morphological mutants are employed. Albino-1 (4637T) and albino-2 (15300) have been routinely used in our laboratory as morphological markers in conjunction with nutritional mutants used in heterokaryotic cultures. These complementary nonallelic albino mutants, when present in different nuclear components of the heterokaryon, impart a normal or near normal light orange color to the conidial mass of the cultures in a variety of nuclear ratios. However, when the heterokaryons have certain disproportionate nuclear ratios in favor of the albino-1 nuclear component, colors of the conidial mass may range from dark yellow to very light yellow or even white in the most extreme ratios. Similarly, in heterokaryons having a very high proportion of albino-2 nuclei, colors range from very light pink to white, the exact color again depending upon the nuclear ratio. The proportion of albino-2 nuclei must be quite high before a color change can be phenotypically recognized as different from the normal wild-type coloration, but a slight excess of albino-1 nuclei can usually be recognized phenotypically. This range in color of the conidial mass of heterokaryotic cultures makes it very easy to detect disproportionate nuclear ratios when albino-1 and albino-2 are present in the respective nuclear components. Although it has not been possible to correlate within narrow limits the nuclear ratios of a culture with its color, and although there is some overlap, it is nevertheless easy to detect the extreme deviate from the normal nuclear ratio in heterokaryons derived from single heterokaryotic conidia (Table 1).

In addition to albino-1 and albino-2, strains of the *nic-2* mutant (4540T) are very useful in heterokaryons because this mutant accumulates a reddish brown pigment in the media when nicotinic acid becomes limiting (BONNER and BEADLE 1946). In a heterokaryon with as much as 95 percent *nic-2* nuclei this color may not be noticeable, but if the nuclear proportion becomes more extreme in favor of the *nic-2* component and the other nucleus cannot supply a sufficient amount of the nutritional requirement, a red-brown to dark yellow pigment is found in

TABLE 1

The relationship between the color of conidial-derived heterokaryotic isolates and the proportion of al-1 *and* al-2 *nuclei in these cultures**

Color phenotype	Number of isolates tested	Proportion of albino-1 or albino-2 nuclei	
		Mean	Range
Wild type	25	.60 *al-1*	.39 *al-1*– .80 *al-1*
Dark-to-medium yellow	13	.82 *al-1*	.58 *al-1*– .91 *al-1*
Light yellow	30	.95 *al-1*	.83 *al-1*–>.99 *al-1*
White (albino-1)	9	.99 *al-1*	.98 *al-1*–>.99 *al-1*
Light pink	16	.93 *al-2*	.79 *al-2*– .98 *al-2*
White (albino-2)	34	>.99 *al-2*	.98 *al-2*–>.99 *al-2*

* The majority of these isolates were from *pan al-1 A* and *nic-2 al-2 A* heterokaryons.

the media. For example, if a heterokaryon between *pan al-1* and *nic-2 al-2* had a nuclear ratio so unequal that the culture had a white phenotype and growth was submaximal, one usually could tell whether the *pan al-1* or *nic-2 al-2* component was in excess by the presence or absence of the brown pigmentation in the media. This phenotype has proved extremely useful for detecting change in nuclear proportion during growth of the heterokaryons in growth tubes.

In the experiments described below extensive use was made of color phenotypes to detect nuclear selection that resulted in highly disproportionate nuclear ratios. More specifically, heterokaryotic conidia were isolated from a large variety of different heterokaryons between various complementary nutritional mutant strains marked with albino-1 and albino-2. When conidial-derived heterokaryotic cultures had a wild-type coloration, the nuclear ratios were considered to be within the range expected to occur in individual conidia. On the other hand, when heterokaryotic conidia produced mature cultures, the majority of which deviated significantly from wild-type coloration, such heterokaryons were investigated to determine if nuclear competition was responsible. In most cases phenotypically-white, conidial-derived heterokaryons were not stable and conidia from such white cultures produced normally colored isolates. Certain heterokaryotic cultures, however, had deviant color types that remained stable, and the *I-i* system described below was discovered in that way.

EXPERIMENTAL RESULTS

Growth behavior of I-i *heterokaryons:* In order to illustrate the interaction of an allelic pair of genes controlling nuclear selection, the growth behavior of various heterokaryotic combinations between the strains described below will be examined. The action of these allelic genes has been studied in a variety of genetic backgrounds, but the salient features of *I-i* interaction can be adequately demonstrated by using only a few strains. Although we have also used mutant strains of *lys-3* (4545), *tryp-1* (10575) and *nic-1* (3416) in combination with *al-1*, *al-2*, *I* and *i*, the behavior of *I* and *i* are described primarily with reference to the following strains: (1) *pan al-1 23A I*, (2) *nic-2 al-2 A I*, (3) *pan al-1 3A I*, (4) *nic-2 al-2 A i*, and (5) *pan al-1 24 A i*. The *pan-1* allele used in these studies was 5531, the *nic-2* allele was 4540T, and *al-1* and *al-2* were 4637T and 15300, respectively (BARRATT, NEWMEYER, PERKINS and GARNJOBST 1954). The designation *nic i*, *nic I*, *pan i*, *pan I*, or simply *I* and *i* often are used, but the reader should bear in mind that the *nic* strains always were marked with *al-2*, and the *pan* strains always carried the *al-1* marker. Strains 1 and 2 above have been used for a good many years in our laboratory, but strains 3, 4, and 5 recently have been recovered from the progeny of crosses of strains 1 and 2 to a standard wild-type strain 74-OR-8-1a. This strain is referred to as 74a and was kindly furnished by DR. F. J. DESERRES. It is also important to note that all of the strains above (except 74a) were carefully selected because they are heterokaryotically compatible with one another and with common tester strains employed in our laboratory.

In the absence of incompatibility factors it has been generally observed that heterokaryons of the same mating type grow at normal rates (BEADLE and COON-

RADT 1944) and that the proportions of the two nuclear components show no consistent change during growth (PITTENGER and ATWOOD 1956). Heterokaryons between two *I* strains, for example, *pan al-1 A I* and *nic-2 al-2 A I*, have these attributes in a wide variety of nuclear ratios (Table 2A). By using different input ratios of the two nuclear types (for methods see PITTENGER, KIMBALL and ATWOOD 1955) one can produce heterokaryons that have a high proportion of either of the two nuclear components. Nuclear ratios in such heterokaryons show no systematic change and the rate of growth is normal unless nuclear proportions are extremely disparate (Table 2A). The nuclear ratios in all such cultures were determined by the method described by ATWOOD and MUKAI (1955) and a $\overline{n}$ value of 2.5 was used in calculating all such ratios.

The growth behavior of such heterokaryons may vary widely, however, when heterozygosity of *I* and *i* is involved. Consider an experiment (results presented in Table 2B) in which *pan al-1 A I* and *nic-2 al-2 A i* were used. When a heterokaryon with a high proportion of *nic i* nuclei was prepared the growth was constant at wild-type rates and the nuclear ratios at the proximal and distal ends of the growth tubes showed no systematic change in favor of *I* or *i* nuclear types. On the other hand, when the input ratio of *I* to *i* was approximately 1:1, or when *I* nuclei were in excess, the behavior of the heterokaryons differed widely from the behavior of those with *i* in excess. The behavior of these heterokaryons (Table 2B) suggests a nonadaptive nuclear change. The heterokaryons were nearly white, indicating extreme nuclear disproportion favoring one of the albino components, and they grew only a short distance (from 55 to 170 mm) before stopping completely. The nuclear ratios, determined from the conidia produced in the proximal ends of these growth tubes, showed the cultures to be essentially homokaryotic for the *pan al-1 A I* nuclei, although a few heterokaryotic conidia were recovered. When conidia could be obtained from these cultures after growth had stopped, they were homokaryotic for *pan al-1 A I*. It is hypothesized that such cultures stopped growing because they had become homokaryotic.

The essential neutrality of the *pan* and *nic-2* markers in the system can be shown by noting the behavior of the heterokaryon between *pan al-1 24A i* and *nic-2 al-2 A I* with the *I* and *i* reversed in regard to the nutritional and albino markers. Heterokaryons with high proportions of *pan i* nuclei had normal rates of growth with no significant systematic changes in nuclear ratios during growth (Table 2C). On the other hand, heterokaryons with initial high proportions of *nic-2 I* nuclei always stopped before reaching the end of the growth tubes; the media showed a dark brown discoloration throughout the area of growth indicating a very high proportion of *nic-2 I* nuclei. Analysis of conidia produced at the proximal ends of such growth tubes showed the cultures to be essentially homokaryotic for the *nic-2 I* component. When the initial mixing ratio was approximately 1:1, growth was normal for the first 24–36 hours. Growth then slowed and the dark brown discoloration of the media accompanied the slower rate. However, such cultures reached the ends of the 500 mm-long growth tubes. Frequencies of *nic-2 al-2 I* nuclei at the proximal ends of the growth tubes ranged from 93 to 99 percent. At the distal ends of the growth tubes only *nic-2 I* homo-

TABLE 2

The growth behavior and the nuclear proportions of various I-I, i-I, *and* i-i *heterokaryons grown in 500 mm-long growth tubes on Fries minimal medium at 30°C*

Heterokaryon and the approximate initial mixing ratio*	Proportion of specified nuclear types in opposite ends of growth tubes		Growth behavior
	Proximal	Distal	
A. *pan al-1 A I + nic-2 al-2 A I*			
100 *pan I*:1 *nic-2 I*	.95 *pan I*	.95 *pan I*	constant, but submaximal
1 *pan I*:1 *nic-2 I*	.64 *pan I*	.53 *pan I*	normal†
1 *pan I*:100 *nic-2 I*	.07 *pan I*	.06 *pan I*	normal
	.04 *pan I*	.04 *pan I*	normal
	.03 *pan I*	.12 *pan I*	normal
B. *nic-2 al-2 A i + pan al-1 23A I*			
100 *nic-2 i*:1 *pan I*	.86 *nic-2 i*	.78 *nic-2 i*	normal
	.93 *nic-2 i*	.94 *nic-2 i*	normal
	.96 *nic-2 i*	.95 *nic-2 i*	normal
1 *nic-2 i*:1 *pan I*	.01 *nic-2 i*	.00 *nic-2 i*	stops‡
1 *nic-2 i*:100 *pan I*	.01 *nic-2 i*	.00 *nic-2 i*	stops
C. *pan al-1 A i + nic-2 al-2 A I*			
10 *nic-2 I*:1 *pan i*	.93 *nic-2 I*	1.00 *nic-2 I*	stops
	.99 *nic-2 I*	1.00 *nic-2 I*	stops
	.98 *nic-2 I*	1.00 *nic-2 I*	stops
1 *nic-2 I*:1 *pan i*	.98 *nic-2 I*	1.00 *nic-2 I*	labile and submaximal§
	.95 *nic-2 I*	1.00 *nic-2 I*	labile and submaximal
	.93 *nic-2 I*	1.00 *nic-2 I*	labile and submaximal
1 *nic-2 I*:10 *pan i*	.90 *pan i*	.91 *pan i*	normal
	.88 *pan i*	.98 *pan i*	normal
	.71 *pan i*	.72 *pan i*	normal
D. *pan al-1 24A i + nic-2 al-2 A i*			
	.89 *pan i*	.90 *pan i*	normal
	.78 *pan i*	.84 *pan i*	normal
	.73 *pan i*	.67 *pan i*	normal
E. *lys-3 al-1 10a i + nic-1 al-2 22a i*			
	.93 *nic i*	.93 *nic i*	normal
	.83 *nic i*	.91 *nic i*	normal
	.85 *nic i*	.95 *nic i*	normal
F. *lys-3 al-1 10a i + nic-1 al-2 10a i*			
	.83 *nic i*	.84 *nic i*	normal
	.86 *nic i*	.85 *nic i*	normal
	.83 *nic i*	.85 *nic i*	normal

* When heterokaryons were prepared by superimposing conidia of the two components, no data were available for initial mixing ratios.
† Normal indicates growth was constant and ca. 4 mm/hr.
‡ Stops is interpreted to mean heterokaryon has become homokaryotic.
§ Culture probably would have stopped if growth tubes had been longer.

karyotic conidia could be recovered, though *pan i* nuclei should have been recovered even if only one in 10^6 was present. It seems clear that although these mixtures were initially heterokaryotic, they became homokaryotic as growth

proceeded and would have stopped had growth not been limited by the length of the growth tube.

The data presented indicate that normal growth and constancy of nuclear ratios in *I-i* heterokaryons depend on the initial ratio of *I* to *i* nuclei. Furthermore, Table 2 data suggest that when *i* nuclei are present in proportions greater than 70 percent, the nuclear ratios and growth rates are constant, or at least show no systematic changes. On the other hand when the proportion of *i* nuclei is less than 70 percent, the nuclear ratios change rapidly in favor of *I* nuclei, the nuclear mixture rapidly becomes homokaryotic in favor of *I* nuclei, and growth stops for lack of the growth factor needed by the now homokaryotic *I* nuclear component.

Will heterokaryons initially with high enough proportions of *i* nuclei to insure normal growth eventually show an increase in *I* nuclei? Such behavior has not yet been observed. For example, in one experiment a *pan al-1 24A i* strain was combined with a *nic-2 al-2 A I* strain with an initial frequency of 92 percent *pan i* nuclei. The heterokaryon was grown for one month in a continuous growth tube without being transferred and the nuclear proportions were regularly determined 12 different times. Although fluctuations in the nuclear ratio occurred during growth, the proportion of *pan i* nuclei at the end of one month's growth was 96 percent. The significance of this increase in *i* nuclei was reduced by fluctuations almost twice as great in the opposite direction during growth.

Many heterokaryons between *i* strains have been examined. The behavior of three are presented in Table 2 to show the variation encountered. Some nonsignificant (t test) changes in nuclear ratios were found. Thus, observations of *i-i* and *I-I* combinations have shown no systematic change in nuclear proportions like those found in *I-i* combinations.

The behavior of conidial-derived heterokaryotic cultures from I-i *combinations:* The demonstrated nonadaptive increase of *I* nuclei in heterokaryotic combination with *i* nuclei, should make possible the prediction of the growth behavior of cultures derived from heterokaryotic conidia. From the preliminary results presented, one would predict that heterokaryons with 70 percent or more *i* nuclei should be capable of normal growth. Expressing this same relationship in whole numbers, any conidium with a ratio of 3 *i*:1 *I* or greater should give rise to a culture with normal growth and most should have normal or near normal coloration. On the other hand, all heterokaryotic conidia with a greater proportion of *I* to *i* nuclei should show a nonadaptive increase in the *I* component and be nearly white from nuclear disproportion.

Analyses of unpublished data by PITTENGER and ATWOOD on the number of nuclei per conidium from a variety of different heterokaryons grown on minimal medium, including nuclear determinations of over 41,000 conidia, showed only 13.7 percent of the conidia with four or more nuclei. In an *I-i* heterokaryon, the percentage of such multinucleate conidia that are heterokaryotic is unknown, as is the percentage with ratios of *i* to *I* favorable to normal growth. Conceivably both percentages could be very small. Conversely one would expect a large percentage of heterokaryotic conidia to have ratios of *I* to *i* within the range where

I nuclei could inhibit *i* nuclei. Such cultures should have high proportions of *I* nuclei and abnormal color phenotypes.

In the course of this investigation thousands of heterokaryotic conidia were isolated from a variety of *I-I*, *I-i*, and *i-i* combinations which were heterozygous for *al-1* and *al-2* as well as for two different nutritional markers. The number of such isolates with wild-type coloration were compared with the number with colors indicating extreme nuclear disproportion. Classifying conidial-derived cultures according to color phenotypes has been the most satisfactory way to distinguish *I-i* heterokaryons from either of the homozygous combinations. A tabulation of some typical results is given in Table 3 and summarized as follows: When a heterokaryon is homozygous for either *I* or *i*, nearly all combinations analyzed showed that a majority of the cultures derived from single conidia had normal wild-type coloration, or the color was within the range expected from ratios known to occur regularly in conidia. Some exceptions, discussed later, were found; however, they most certainly resulted from factors other than *i*. If a heterokaryotic culture is isolated from an *I-I* or *i-i* combination and found to have a high proportion of one of the nuclear components, conidial-derived heterokaryotic isolates recovered from this culture usually will exhibit the normal color phenotype. In other words, these deviant types are not stable.

TABLE 3

The percentage of conidial-derived heterokaryotic isolates with normal color from various al-1 *and* al-2 *heterokaryons*

Heterokaryon	Number of isolates tested	Percent normal*	Percent abnormal
	I-I combinations		
pan al-1 23A I + nic-2 al-2 A I	232	76.0	24.0
nic-1 al-2 88a I + lys-3 al-1 a I	100	100.0	0.0
pan al-1 3A I + nic-1 al-2 55A I	145	100.0	0.0
	I-i combinations		
tryp-1 al-1 a i + nic-1 al-2 88a I	10	10.0	90.0
nic-2 al-2 A i + pan al-1 3A I	194	3.6	76.4
lys-3 al-1 10a i + nic-1 al-2 88a I	15	0.0	100.0
nic-2 al-2 A i + pan al-1 23A I	55	16.0	84.0
nic-2 al-2 A i + lys-3 A I	20	15.0	85.0
pan al-1 24A i + nic-2 al-2 A I	90	26.0	74.0
nic-1 al-2 22a i + lys-3 al-1 a I	64	3.1	96.9
	i-i combinations		
pan al-1 24A i + nic-2 al-2 A i	20	85.0	15.0
lys-3 al-1 10a i + nic-1 al-2 22a i	15	100.0	0.0
lys-3 al-1 10a i + nic-1 al-2 10a i	15	100.0	0.0
lys-3 al-1 10a i + nic-2 al-2 a i	20	95.0	5.0
lys-3 al-1 22a i + nic-2 al-2 a i	15	100.0	0.0
lys-3 al-1 18a i + nic-2 al-2 a i	14	100.0	0.0
lys-3 al-1 27a i + nic-2 al-2 a i	15	93.3	6.7
lys-3 al-1 29a i + nic-2 al-2 a i	15	100.0	0.0

* Normal means any phenotype expected on the basis of the nuclear ratios known to be normally present in conidia; dark to medium-yellow phenotypes were classified as normal.

The situation is quite different when heterokaryotic colonies are isolated from *I-i* combinations (Table 3). In such heterokaryons, regardless of the nuclear ratios present in the "parent heterokaryon," the majority of the conidia from such combinations give rise to new cultures having proportions of *I* nuclei far in excess of the nuclear proportions present in individual conidia. When *I-i* conidial-derived cultures were not white, they usually had a high proportion of *i* nuclei. The degree of nuclear disproportion attained in small test tube slants depended somewhat upon the nutritional mutants involved. For example, heterokaryotic colonies between *nic-2* and *pan* usually had a greater proportion of *I* nuclei when the *nic-2*, rather than the *pan* component, was *I*. The behavior of conidial-derived *I-i* cultures can also be determined further by observing the growth of heterokaryotic colonies transferred to growth tubes rather than to slants. Since the results are according to expectations, a detailed presentation of the data is not warranted. It will suffice to say that some single heterokaryotic colonies, when transferred to 500 mm-long growth tubes, were normal in color and growth rate, and the frequency of *i* nuclei in such cultures was always over 70 percent. The majority of the isolates, however, grew for varying distances but growth in the tubes always ceased as a result of homozygosity of the *I* component. Thus, the expected nonadaptive increase in the *I* nuclei was observed except in cases where the initial frequency of *i* nuclei was high enough to insure normal growth.

Descriptions of other I-i *and* i-i *interactions in various heterokaryotic combinations:* The *pan al-1 24A i* and *nic-2 al-2 A i* strains used extensively in the previous sections of this report were selected because the presence of *i* in them has been verified by its segregation in the progeny of crosses to *I* strains. Also a number of *i* strains have been isolated by crossing *I* strains to both ST 74A and 74a and also by crossing *nic-2 al-2 A i* with *lys-3 al-1 a I*. The progeny of these crosses have provided a large number of additional isolates that have been tested in many heterokaryotic combinations. Some are shown in Table 3. For example, *nic-1 al-2 88a I* and *nic-1 al-2 55A I* have been used extensively as *I* tester strains with many *i* isolates of various types. By crossing these two strains with 74A and 74a, it has been possible to recover *nic-1 al-2* strains (see *nic-1 al-2 22a i* and *nic-1 al-2 10a i*, Table 3) among random isolates which were *i*, demonstrating that both of these wild-type strains carry the *i* gene. That many mutants in common usage have been induced in 74A backgrounds and still form normal heterokaryons is ample evidence that *i-i* heterokaryotic combinations can show normal heterokaryotic behavior. That *i-i* combinations not in the more uniform 74A background also may behave normally is shown by data in Tables 2 and 3. Nevertheless, other genetic factors clearly affect *i-i* and *I-i* interactions because some such combinations fail to show the expected phenotypes. Nearly all of the exceptional phenotypes encountered involve one strain, *lys-3 al-1 a I*, or strains which were recovered from progeny of crosses in which this strain was one of the parents. For example, in a heterokaryon between *lys-3 al-1 a I* and *nic-1 al-2 22a i*, the *I* component undergoes the expected increase in proportion during growth until approximately 70 percent of the nuclei are *I* and then

no further increase in the *I* component takes place. Such heterokaryons, however, were white rather than the expected wild-type color when this equilibirium in the nuclear ratio was reached and the growth rate was constant but approximately half maximal. As an illustration, the behavior of one such heterokaryon follows. Conidia were initially mixed in a ratio of nine *nic-1 al-2 22a i* to one *lys-3 al-1 a I* and the conidial mixtures placed directly into a 1000 mm-long growth tube with nine sampling ports along its top at 100-mm intervals to facilitate removal of conidia to be used in the determination of nuclear ratios. The percentages of *lys al-1 a I* nuclei from these nine sampling ports, starting from the proximal end, were 30, 57, 64, 72, 73, 73, 69, 69, and 71. The conidia formed below the first three sampling ports had approximately normal wild-type color, below the fourth they were light yellow, and thereafter they were white. Initially the growth rate was 4 mm/hr, but approximately a third of the way down the tube growth slowed to ca. 2.0 mm/hr and remained constant thereafter. Similar results have been found using other *i* strains in combination with this one *lys* strain. In a cross of this same *lys*-requiring strain with *nic-2 al-2 A i*, approximately half of the *lys-3 al-1 a i* isolates behaved as one would predict in combination with several *i* and *I* strains but the remainder showed a variety of unexpected results in combination with both *i* and *I* testers. The genetic analysis of these deviant cases has not yet been completed, but it seems clear that the expected interaction betwen *i* and *i*, or *I* and *i* genotype may not be manifested in all cases probably because of the residual genotypes of the nuclei involved. Initially, attempts have been made to describe the phenotypic interactions which seem paramount without analyzing each deviant case, however, attention should be called to the deviant behavior if only to emphasize that other genetic factors often may mask the *i-I* interaction or perhaps be epistatic to these alleles.

Inheritance studies: Because inheritance studies of a gene whose phenotype can only be detected in heterokaryons is extremely laborious and complicated by the large number of tester strains needed, our data regarding the inheritance of *I* and *i* are not extensive. The results, however, appear to be straightforward and indicate that *i* and *I* are determined by a single factor which is located either on linkage group I or II .

The *I* or *i* genotype of a particular strain was determined by making a heterokaryon between the unknown strain and the appropriate *I* and *i* tester strains. Most of these heterokaryons were then placed in growth tubes and their behavior observed. Heterokaryotic conidia from each tested combination were isolated as colonies from minimal plates and placed on minimal agar slants. Color phenotypes were then used in conjunction with their growth behavior in growth tubes to determine the genotypes of the strains.

Since both *al-1* and *al-2* are needed in tests to simplify the identification of *i* and *I*, the segregation of these mutants was analyzed in a cross of *pan al-1 A I* and *nic-1 al-2 -10a i*. Although use of *al-1* complicates the analyses somewhat since it is inseparable from a reciprocal translocation involving linkage groups I and II (BARRATT *et al.* 1954), the use of this marker turned out to be quite fortunate.

Asci with eight normal spores were collected as unordered tetrads by the method suggested by STRICKLAND (1960). Such asci have no single crossovers in either interstitial segment of the translocation heterozygote and result from alternate segregation of the ring-of-four chromosomes at metaphase I. Analysis of 13 asci showed a 1:1 segregation of *I* to *i* in each ascus. Since the analysis of unordered tetrads gives no evidence of centromere distance and since the data is not extensive enough to definitely prove the location of *i*, the genotypes of each spore pair will not be enumerated here. It will suffice to mention that 16 *pan* isolates were *I* and 10 were *i* suggesting independent segregation of *pan* and *I*. However 18 of the *nic* isolates were *i* and 8 were *I*. Likewise 20 *a* isolates and 20 *al-2* isolates were *i*, and only six *A* and six *al-2* isolates were *I*. In a similar cross (*nic-1 al-2 a I* × *pan al-1 A i*) in which the *nic* marker came into the cross with *I*, 20 of the 24 random *nic*-isolates were *I* and only four were *i*. Although these results clearly show an association of the markers on linkage group I and the *i* locus, they do not prove that the locus is on linkage group I. The apparent ambiguous results are due to the pseudolinkage brought about by the association of *al-1* with linkage groups I and II as a result of the reciprocal translocation. On the basis of the limited tetrad data available, DR. DAVID PERKINS, who was good enough to examine the data, has suggested that linkage group II is an even more likely site of *i* than is linkage group I. However, the data are insufficient to warrant any definite conclusion and a detailed presentation of the data will be deferred until a more exact location of *i* is determined.

DISCUSSION

The experimental results clearly show that when *I* and *i* nuclei are present in a heterokaryon the proportion of the *I* nuclear component may undergo a nonadaptive increase which eventually leads to homozygosity of the *I* nuclei. Although it is possible to demonstrate under what conditions the increase in *I* nuclei takes place, it is not clear what mechanism is responsible for the increase. Several obvious possibilities were examined. An explanation based on heterokaryotic incompatibility of the type described by GARNJOBST and WILSON, (1956) and WILSON *et al.* (1961) was dismissed when it was found that *I-i* combinations could grow normally in certain ratios. If there is a direct relationship in heterokaryons between nuclear and cytoplasmic proportions, as suggested by certain experiments involving a mixture of a normal cytoplasm with a cytoplasmic mutant (PITTENGER, unpublished), then it is quite probable that the cytoplasm can be ruled out as having a controlling function in the division of *i* nuclei. It is known that conidial-derived cultures show a nonadaptive increase of *I* nuclei regardless of whether the conidia were formed by a culture with a high proportion of *i* nuclei and *i* cytoplasm or by a culture with a high proportion of *I* nuclei and *I* cytoplasm. Consequently, it appears that the cytoplasm of the parent culture, which is present in the conidia formed by such a culture, does not have an inherent role in regulating division rates of the two nuclear components.

Certain observations could be interpreted to inidicate that *I* and *i* nuclei have inherently different rates of nuclear division. If such an explanation were true,

however, one would expect always to find a systematic increase in the proportion of *I* nuclei during growth of heterokaryotic cultures. Such an increase in *I* nuclei was not found when the proportion of *i* nuclei was greater than .70. Heterokaryons with over .70 *i* nuclei, observed for over a month in continuous growth-tube cultures, showed no systematic increase in the *I* component. Thus, an interpretation based solely on an inherent difference in division rates of the nuclear components does not explain the data. On the other hand, a possible explanation for such behavior, suggested by PONTECORVO (1946) on theoretical grounds, is that nuclei of different kinds within a heterokaryon multiply at different rates, the rates depending on the proportion of the two nuclear types. Although such a postulate may explain the interaction of *I* and *i* in heterokaryons and may account for other instances of labile growth rates that have been observed, it is difficult to prove because of an unequivocal way of measuring nuclear division rates.

These observations are somewhat similar to another case of nonadaptive growth described by RYAN and LEDERBERG (1946), and as RYAN (1946) has pointed out, it is not known whether the minority nuclear component is actually destroyed, merely inhibited, or simply left behind as growth proceeds. In the present case, it is clear that *i* nuclei are neither destroyed nor left behind completely, although frequently the change from a heterokaryon to a homokaryon appears to be unusually rapid. Inhibition, at least in an operational sense, seems to be the most likely of the three alternatives. For example, in a *nic-2 al-2 A i* + *pan al-1 A I* heterokaryon grown in a growth tube with sampling ports, it was found that 25 mm from the onset of growth there were 47 percent *i* nuclei; at 100 mm only 11 percent *i* nuclei were found and at 250 mm no *i* or heterokaryotic *i-I* conidia were found among the 3690 viable conidia plated. Since the number of *i* nuclei estimated to be in the conidia alone at the two sampling ports is far in excess of the number added at the beginning of the growth tube, it is clear that they divided in the presence of *I* nuclei, but apparently at a rate different from *I* nuclei. Likewise cultures derived from heterokaryotic conidia may have very disproportionate numbers of *I* nuclei, and still have far in excess of the actual number of *i* nuclei present in the original conidium. Consequently, *i* nuclei can divide in the presence of *I* nuclei undergoing a nonadaptive increase in number. Growth rates and purely practical considerations, however, lead one to believe that it is the division rate of *i*, rather than *I*, nuclei that is altered, at least until the growth of *I* nuclei in the balanced heterokaryon is limited by the availability of the growth factor supplied by *i* nuclei. At the same time it appears reasonable to assume that different proportions of *I* nuclei might have different effects on the rate of multiplication of *i* nuclei.

Although it appears that the multiplication of *i* nuclei is inhibited in the presence of excess *I* nuclei, that *I* nuclei produce any substance capable of inhibiting or altering the division rate of *i* nuclei as measured by growth rates of *i* cultures has not been demonstrated in this laboratory. For example, homokaryons of *nic-2 al-2 A i* and *nic-2 al-2 A I* strains have equal growth rates as measured in growth tubes and as determined by dry weight of mycelia from

liquid shake cultures. *i* strains grow at the same rate in growth tubes supplemented with autolysates of either *I* or *i* strains. No effect on growth was noted when growing *i* cultures in sterile filtrates of liquid medium taken from shake cultures or *I* and *i* cultures. If the mycelia of *I* isolates are homogenized and added to wells in agar plates, the homogenates fail to inhibit the growth of *i* conidia seeded on the plates. Although every attempt to show that *I* strains inhibit the growth of *i* strains has failed, such an inhibitory substance may exist and be difficult to demonstrate because of the weakly dominant condition of *I* over *i*. Likewise growth rate in Neurospora need not show a good correlation with rates of nuclear division.

Though the possibility of an inhibitor's being involved in the interaction of *I* and *i* nuclei has not been completely ruled out, there is no compelling reason to prefer this explanation over others. For example, the data can be interpreted to indicate that the interaction of *I* and *i* involves competition for a substrate, conceivably one necessary for nuclear replication. Under this hypothesis *i* nuclei could successfully compete for such a substrate only when the proportion of *i* nuclei was greater than approximately .70. If an excess of such a substrate could be added to the growth media, normal growth of both nuclear types might be expected over a wide range of nuclear ratios. At present, however, such a hypothesis is unsupported because no supplement has been found that will prevent the nonadaptive increase of *I* nuclei. 1:1 mixtures of *I* and *i* conidia in two different heterokaryotic mixtures showed the usual increase of *I* nuclei on several different concentrations of yeast extract, vitamin mixtures, and amino acid mixtures as well as on single supplements of the sulfur-containing amino acids. Supplementing the medium with the growth factor of the *i* nuclear component had no stabilizing effect on the nuclear ratios. Likewise, supplementation with various DNA precursors has given only negative results. A search is continuing for some clue to the actual mechanism involved, whether it be inhibition, substrate competition or some other metabolic control system. But the operational behavior of these alleles in heterokaryotic combination is our only clue so far.

Heterokaryotic systems in Neurospora provide an excellent tool to determine dominance of the wild-type alleles of nutritional mutants, but very little work has been done on this problem. Beadle and Coonradt (1944) were able to show that the normal allele of *pan-1* (5531) was strongly dominant. Pittenger and Atwood (1956) refined that discovery by showing that only 3–4 percent of the *pan*$^+$ allele was needed to give normal growth. In comparison to the above case it would appear that *I* is only weakly dominant to *i*. Several attempts have been made to determine the exact proportion of *i* nuclei needed to prevent the increase in *I* nuclei during heterokaryotic growth, but this proportion has yet to be pinpointed. That is, it has not been demonstrated, for example, that a heterokaryon with 70 percent *i* nuclei will have a stable nuclear ratio whereas in the same experiment one with slightly less than 70 percent will show an increase during growth of *I* nuclei. Although frequencies of *i* nuclei approaching, but less than, 70 percent are undoubtedly formed, the rapid increase in *I* nuclei probably prohibits detection of such ratios. Nevertheless, a knowledge of the approximate

proportion of *i* nuclei needed for stable nuclear proportions during growth has been extremely useful not only in interpreting the growth behavior of heterokaryotic conidia, but also in explaining the exceptional types. Conidial-derived *I-i* heterokaryotic cultures with the normal wild-type coloration are routinely found in small proportions in certain *I-i* heterokaryons but are very rare in others. When present, they are almost invariably found to have a high enough proportion of *i* nuclei to insure normal growth and color. When heterokaryotic conidia from such cultures are isolated, they again give rise to cultures, the majority of which have a high proportion of *I* nuclei. Regardless of the explanation of the origin of such cultures, the important thing is that this phenotype usually is unstable.

The study of *I* and *i* in heterokaryotic combinations has not only demonstrated the importance of nuclear proportions in the expression of nuclear competition resulting in incompatibility, but also has emphasized the difficulties encountered in attempting to characterize compatibility and incompatibility. If heterokaryotic compatibility is provisionally defined as the ability of different nuclei to coexist in harmony in the same cytoplasm, then a compatible heterokaryon would be phenotypically recognized as one capable of growing at a constant rate comparable to the fastest growing nuclear component. Any heterokaryotic culture deviating from this norm would be considered incompatible. Nuclear competition resulting in homozygosity would thus be considered an incompatible reaction. The interaction of *I* and *i* demonstrates the difficulties inherent in such a definition since such a combination would appear to be either compatible or incompatible depending on the nuclear ratio present. A similar problem can exist in any forced heterokaryon which might exhibit a submaximal growth rate because of nuclear disproportion rather than from any inharmonious interaction between the nuclei. These two extremes emphasize the necessity of adequate testing to determine the phenotypic potentials of any heterokaryotic combination. Although trying to fit the behavior of these allelic genes into a preconceived notion of compatibility is largely a matter of semantics, certain ambiguous results in our studies also emphasize the difficulty of trying to make all of our observations conform to current thinking regarding the mechanism of genetic control of compatibility. The best evidence available on this point is the work of GARNJOBST (1955) on the *het*⁻ factors, *C* and *D*, and their alleles. Her studies revealed that homozygosity of these genes is necessary for the formation of a stable, compatible heterokaryon. Many other cases of incompatibility (see particularly HOLLOWAY 1955) also have been shown to involve heterozygosity of certain genes. Apparently it has, therefore, been generally assumed that homozygosity of genes controlling heterokaryon compatibility is a prerequisite for normal heterokaryotic growth. This generalization is obviously true as far as it goes because in the absence of heterozygosity there are no heterokaryons. However, what kinds of genes affect heterokaryotic compatibility and must be homozygous remains a question. Continued study may show that heterozygosity of almost any gene, in the proper genetic background, has the potential of affecting compatibility. For example, in the present studies we have observed that two

different *i* isolates give the expected phenotypes with a common *i* and *I* tester, but with a different *I* tester one of the *i* strains gives the expected phenotype and the other does not. This suggests that the residual genotype of the *I* and *i* strains may either enhance or prevent the full expression of these genes. These observations and others more pertinent noted below suggest that some genes may be responsible for incompatibility in some combinations but have no effect in other combinations. That is, compatibility may involve not only interaction between alleles, but also interaction between nonalleles.

Returning to the question of the kind of genes that affect compatibility, the study of RYAN and LEDERBERG (1946) demonstrates that a gene which functions in the synthesis of an essential amino acid may also affect incompatibility. They showed that on leucine-limiting medium a nonadaptive increase of a leucine-requiring nucleus took place when it was in heterokaryotic combination with a wild-type strain which had arisen by back mutation of the leucine mutant. Thus they demonstrated a nonadaptive increase in one nuclear type which presumably differed from the other nuclear component by a single gene. Hence this incompatibility is attributable to heterozygosity of a single gene not ordinarily thought of as influencing compatibility. On the other hand, RYAN (1946) reported that the nonadaptive increase of the leucineless mutant failed to take place when the wild-type component of the heterokaryon did not arise by reversion of the leucine mutant. Since these two wild-type strains appear to be different in their residual genotypes and at the leucine locus as well, several interpretations of the results are possible. One is that the intra-allelic interaction between the nutritional mutant and the respective isoallelic wild-types may result in compatibility or incompatibility, depending upon the particular allele involved. A second is that allelic genes which usually interact to give an incompatible phenotype might also interact with certain nonallelic genes to give a compatible phenotype or *vice versa*. The results also suggest that certain ambiguous cases of noncomplementation in intra-allelic heterokaryons may be caused by a similar type of incompatibility. DR. VAL WOODWARD (personal communication) has recently shown us some very interesting unpublished data in support of such a notion. His results have shown that (1) nonallelic mutation changes the complementation pattern, (2) the new mutation is specific to a given pair of alleles (in a series of over 50 heteroalleles), and (3) the pattern is the same as that of nonallelic compatibility.

That particular genes may affect compatibility in one genotype and not in another also can be inferred from the studies of DAVIS (1960 a,b). He found that gene *m* affects the ability of *pan-1* strains to grow on limiting amounts of pantothenate. In a heterokaryon between two nonpantothenate-requiring strains the gene *m* in a heterozygous or homozygous condition would presumably have no effect on compatibility but when *m* is present in only one strain of a heterokaryon homozygous for *pan-1*, it results in a nonadaptive increase of the m^+ *pan-1* component. Thus it is apparent that the gene *m* in a heterozygous condition would have a very different effect on the interaction of the two nuclear

components, depending on whether the two nuclei making up the heterokaryon were homozygous for *pan-1* or homozygous for the normal allele of *pan-1*.

Although these two isolated instances, along with certain observations of *I-i* interactions mentioned earlier, may not constitute convincing evidence in support of the contention that specific genetic factors may be compatible in one genotype but incompatible in another, considerable evidence appears to be irrefutable in this regard. HOLLOWAY (1953) has shown that three strains 5531A (*pan*), 1633A (*pab*), and 4545A (*lys*) form stable compatible heterokaryons in all possible combinations with one another. He then crossed the *pab* strain to wild-type Abbott 12a and selected 55 different *pab A* isolates. These were then tested for compatibility with the original *pan A* and *lys A* strains. Thirty-seven of the *pab* reisolates were compatible with *pan A* and only two were compatible with *lys A* even though *lys A* and *pan A* are compatible with one another. When *lys A* was crossed with Abbott 12a, 49 *lys A* reisolates were recovered. Only one was compatible with *pab A* and only one, with *pan A*. Twenty-eight reisolates of *pan A* were recovered from a cross to Abbott 12a; 13 were compatible with *pab A* and none, with *lys A*. Although these data were originally presented to support the contention that heterokaryotic compatibility was under genetic control, they are now even more meaningful in another regard. These results make it perfectly clear that two strains' being compatible with one another does not insure that a third strain compatible with the first will necessarily be compatible with the second. Such behavior may mean that compatibility is often strain specific and that heterozygosity of certain genes may have no effect on compatibility in one genotype, but in another such a condition will result in incompatibility.

[ADDENDUM: Since the findings of HOLLOWAY (1953) are especially pertinent to an understanding of the genetic control of compatibility, we have repeated the same type of experiments after our manuscript was submitted. Different strains have had to be used and what we consider to be more reliable techniques for detecting incompatible heterokaryotic combinations have been utilized. Our results are different from HOLLOWAY's for in rather large scale experiments we invariably have found that when two strains are compatible with one another, both are always either compatible or incompatible with a third strain; that is, two compatible strains have never reacted differently with the same tester strain.]

Since the interaction of *I* and *i* may result in incompatibility, their relationship to other genes affecting heterokaryon compatibility is of interest. All of our *I* and *i* strains of mating type *a* are incompatible with the *CD*, *Cd*, *cD* and *cd* testers kindly furnished by DR. LAURA GARNJOBST. Since their incompatibility phenotype is also different there in every reason to believe that different loci are involved. The nonadaptive increase of *I* under certain conditions is reminiscent of the unstable *Y-Y'* heterokaryon reported by HOLLOWAY (1953, 1955). We have been unable, however, to find any evidence that *Y-Y'* combinations show normal growth under any conditions and for this reason alone it must be assumed that this system is different from *I-i*, although it is conceivable that they could be the same.

SUMMARY

1. An allelic pair of genes, designated *I* and *i*, have been found whose only known phenotype is expressed when the genes are present in a heterokaryon in a heterozygous condition. When certain nuclear proportions are present in forced heterokaryons, *I* nuclei operationally inhibit the multiplication of *i* nuclei with the result that *I* nuclei undergo a nonadaptive increase leading to homozygosity of this nuclear component.

2. The data suggest that *I* is only weakly dominant to *i* because the nonadaptive increase in the *I* nuclear component takes place only when the proportion of *I* nuclei is approximately .30 or greater. When more than 70 percent of the nuclei are *i*, growth is normal for an indefinite period and no systematic change in the nuclear ratio is found. These relationships are found either in heterokaryons prepared with a variety of nuclear ratios, or in cultures derived from single heterokaryotic conidia.

3. At present the interaction of *I* and *i* can be described only in operational terms. The incompatibility of *I* and *i* apparently is not from a cytoplasmic difference, nor can the results be satisfactorily explained by an inherent difference in the rates of nuclear division of the two types. It has not been possible to demonstrate that *I* nuclei produce a substance which inhibits the division of *i* nuclei or that their interaction is from competition for a substrate conceivably needed for nuclear replication.

4. The behavior of *I* and *i* has been satisfactorily studied using *nic-1*, *nic-2*, *pan-1*, *lys-3*, and *tryp-1* in forced heterokaryons which suggests that the interaction of these alleles is independent of the nutritional mutants present. However, some *i-i* and *I-i* heterokaryons do not show the expected phenotypes, indicating that their interaction is not completely independent of the residual genotypes of all of the strains used.

5. Inheritance studies show that *i* segregates as a single genetic factor in crosses and is located either on linkage group I or II. *i* has been found in wild-types ST 74A and 74-OR-8-1a.

6. The interaction of genes effecting compatibility in heterokaryotic systems is discussed, along with their relationship to problems involved in the study of complementation phenomenon.

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DISTRIBUTION OF "SEX-RATIO" AGENT IN TISSUES OF DROSOPHILA WILLISTONI[1]

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Received August 14, 1961

THE abnormal condition referred to as "sex-ratio" in *Drosophila willistoni* is characterized by extreme departures from the normal 1:1 ratio of the sexes, i.e. either completely, or nearly completely, female progenies (MALOGOLOWKIN and POULSON 1957; MALOGOLOWKIN 1958). Absence of males is a consequence of mortality in embryonic stages (MALOGOLOWKIN and POULSON 1957; MALOGOLOWKIN, POULSON and WRIGHT 1959), the time of onset of the disturbance being a function of the genotypic background (POULSON and COUNCE 1959; COUNCE and POULSON 1961). The abnormal dying eggs contain an agent which when introduced into females of previously normal strains converts them to the "sex-ratio" condition (MALOGOLOWKIN and POULSON 1957; MALOGOLOWKIN, POULSON and WRIGHT 1959). The rarely surviving sons of "sex-ratio" females were found to be incapable of transmitting the condition (MALOGOLOWKIN 1958). By analogy with other instances of "sex-ratio" (MAGNI 1954, 1956; CAVALCANTI, FALCÃO and CASTRO 1957) such males have been assumed to be free of the "sex-ratio" agent.

This paper is an account of experiments to test the distribution and localization of the infectious "sex-ratio" agent particularly in the organs and tissues of adult flies as a first step in the elucidation of the nature of the agent. A preliminary report was given by SAKAGUCHI and POULSON 1959.

MATERIALS AND METHODS

The SR strains used in these studies were derived from the original strain studied by MALOGOLOWKIN (1958) and from transferred strains derived by MALOGOLOWKIN, POULSON and WRIGHT (1959). The original SR strain was maintained for many generations by alternate crossings to males from the pink and from the ebony mutant strains and hence was kept heterozygous for these mutants. Such SR females were subsequently crossed to males of a strain, Barbados-3, which had been shown by MALOGOLOWKIN (1958) to be gentoypically favorable for maintenance of the SR condition. At the time when these experiments were begun SR females had been crossed to Barbados-3 males for 18

[1] Supported by a grant from the National Science Foundation (G-6017) which is gratefully acknowledged.

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generations of single female matings. During the latter portion of this time period, and subsequently, the males were derived from a highly inbred line of Barbados-3. This SR strain will be referred to as SR.B-3, the maintainer strain as B-3. Inbred lines of B-3 have always given good 1:1 ratios. The other SR strain was derived from the injection of original SR abnormal male ooplasm into the normal strain Recife-3 (MALOGOLOWKIN, POULSON and WRIGHT 1959) and subsequently maintained by mating with males from the Recife-3 strain. During the latter history of this strain it has been maintained by use of males from a highly inbred strain of Recife-3 origin. This SR strain is referred to as SR3g.R-3 and the maintainer strain as R-3.

For the testing of the various tissues the following procedures were used. In the case of hemolymph small amounts of blood (0.00005–0.00008 ml or 0.05–0.08μl) were removed from the abdomens of donor flies with a micropipet and immediately injected into the abdomens of young virgin females of the recipient strain. In the cases of ovary, muscle, gut, and fat body the individual tissues were removed by dissection from between 50 to 100 donor flies and washed repeatedly in sterile Drosophila Ringer solution. (This is the Waddington solution made up as follows: Solution A contains NaCl 37.5 g, $CaCl_2$ 1.0 g, KCl 0.5 g in 500 ml distilled water; Solution B contains $NaHCO_3$ 1.0 g in 500 ml distilled water. The solutions are autoclaved separately and mixed when needed in the ratio of one part A, one part B, and eight parts sterile distilled water.) The different materials were then homogenized separately in small glass homogenizers in the cold with 0.03 ml sterile Drosophila Ringer solution. The homogenates were allowed to stand for one hour and the supernatant was used as the source of the material injected. As in the case of blood the injected volume was in the range 0.05–0.08 μl/recipient fly. Small glass homogenizers 4 mm $\times$ 30 mm were made from glass tubing and individually fitted with grinding rods. Separate injection pipets calibrated against a standard were used for the handling and injection of the different materials so that there could be no chance of contamination.

Except in the cases of the larvae and the SR males all donors were at least 10–15 days old at the time of the experiments.

Injected females were mated singly to males of their own strain in yeasted creamers containing standard corn-meal-molasses-agar medium and kept at 25°C. The flies were transferred to new creamers every second day. Egg counts were made after the transfer to new creamers. Counts of adults were made from each creamer until emergence was complete.

All instances of probable or suspected SR condition were examined by carrying tests into the second and third generations. It was not possible to test all daughters from all broods in order to estimate latent infections (MALOGOLOWKIN, POULSON and WRIGHT 1959). The instances reported here all made their appearance in the first generation from the injected females.

RESULTS

Adult female tissues: In the tests of the various tissues and organs the most striking results were those obtained in the case of hemolymph, although ovary, muscle, and fat body also gave positive results. Data for the progenies of hemolymph-injected females are presented in Table 1 which is set up so that it may be compared with Tables 1 and 2 of MALOGOLOWKIN, POULSON and WRIGHT (1959). It will be seen that males were totally absent from the progenies of four of the nine females producing adult offspring and that the numbers of males were markedly reduced in four others. In the case of No. 9 there were more males than females. Female No. 10 produced many eggs, but no adults developed. In comparison with the control-injected females, all of which gave 1:1 ratios, the SR-injected females showed a remarkably strong "sex-ratio" effect, for in all except two of the females the effect made its appearance in the very first broods and the unisexual condition persisted through the later broods in every case.

The completeness and stability of the SR condition was tested by single female matings from each of the broods. Data from the tested females are given in Table 2 in which third as well as second generation transmission is strikingly demonstrated. Certain of these lines have since been maintained for many generations. Similar tests, on a more limited scale, from broods 1f, 1g, 3c, 3d, 4c, and 5c among the control-injected females yielded only 1:1 progenies.

Thus the SR agent is not only present in the hemolymph of adult SR females but it is present in such high concentration that the introduction of 0.05–0.08 μl of the SR hemolymph into young normal Barbados-3 females results in its immediate incorporation into their developing eggs and, as seen from Table 2, leads to a large proportion of highly stable SR lines in subsequent generations. In experiments not presented here this has been shown to be true also for the SR3g.R-3 strain.

The results of the tests on other tissues and organs are summarized in condensed form in Table 3. The left-hand side gives the tissues and the age and number of flies from which homogenates were prepared. The middle columns present the results of the injections of the materials from the SR flies in terms of numbers of unisexual progenies resulting and the length of the incubation period before the appearance of the SR condition. The right-hand portion of the table gives the data for control females into which materials from the B-3 normal strain were injected.

Except in the case of the gut, for which the number of surviving recipients was inadequate, the tissues tested gave evidence of the presence of the SR agent. Fat body gave the highest proportion of SR infections. Out of the 13 females producing progenies five gave SR after an incubation period of 10–12 days. The length of the incubation period and the proportion of infections more or less parallel those observed in the case of injection of SR ooplasm by MALOGOLOWKIN, POULSON and WRIGHT (1959). Although completely bathed in hemolymph the fat body possesses a very much lower complement of the infectious agent.

TABLE 1

Progenies from B-3 females injected with blood from SR. B-3 female; and controls injected with B-3 blood

Broods	a		b		c		d		e		f		g		h		i		j		k		l		m		n		o		p		q	
Days	0–2		3–4		5–6		7–8		9–10		11–12		13–14		15–16		17–18		19–20		21–22		23–24		25–26		27–28		29–30		31–32		33–34	
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
SR-injected																																		
1	1	0	0	0	0	0	0	0	3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.	.	.	.
2	0	0	3	2	5	5	7	2	7	1	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.	.	.	.	.	.	.	.
3	5	2	11	0	15	0	14	0	10	0	0	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4	4	0	28	0	7	0	11	0	10	0	4	0	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5	0	1	13	0	31	0	35	0	11	0	12	0	2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	14	0	20	0	23	0	1	0	0	0	0	0	3	0	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
7	0	0	42	25	14	9	1	0	0	0	0	0	0	0	5	0	6	0	0	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.
8	9	0	74	0	34	0	19	0	3	0	10	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.	.
9	0	0	0	2	22	23	5	12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
10	0	0	0	0	0	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Control-injected																																		
1	1	0	43	43	11	17	6	8	1	0	13	9	4	2	0	0	1	0	0	0	0	0	0	0	0	0	.	.	.	.	.	.	.	.
2	1	0	54	51	44	45	49	61	17	19	0	0	2	3	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3	0	0	45	49	3	0	1	3	0	0	0	0	0	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
4	0	0	44	46	34	25	17	7	0	0	0	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5	0	0	50	53	19	20	0	0	0	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

TABLE 2

Second and third generation tests for "sex-ratio" among progenies of SR. B-3 injected females

Injected female	Days after injection		Second generation Daughters tested	Second generation Unisexual progenies	Third generation Daughters tested	Third generation Unisexual progenies
No. 1	a	0–2	1	1	7	7
	e	8–10	2	2	11	11
	f	10–12	1	1	14	14
No. 2	b	2–4	3	0	17	3?
	c	4–6	4	3	32	22
	d	6–8	1	1	9	9
	e	8–10	7	6	24	18
	f	10–12	9	9	17	17
No. 3	a	0–2	4	1	7	2
	b	2–4	10	10	..	..
	c	4–6	4	4	10	10
	d	6–8	8	8	16	16
	e	8–10	7	7	11	11
No. 4	a	0–2	2	0	7	0
	b	2–4	11	11	14	14
	c	4–6	5	5	12	12
	d	6–8	7	7	18	18
	e	8–10	9	9	17	17
	f	10–12	4	4	14	14
No. 5	b	2–4	7	7	18	18
	c	4–6	11	11	24	24
	d	6–8	9	9	16	16
	e	8–10	9	9	15	15
	f	10–12	9	9	12	12
	g	12–14	2	2	9	9
No. 6	b	2–4	8	6	20	14
	c	4–6	9	9	15	15
	d	6–8	8	8	18	18
	e	8–10	1	1	6	6
	h	14–16	2	2	..	..
No. 7	b	2–4	7	5	8	8
	c	4–6	1	1	6	6
	h	14–16	5	5	10	10
	i	16–18	3	3	..	..
No. 8	a	0–2	7	7	10	10
	b	2–4	7	7	7	7
	c	4–6	11	11	3	3
	d	6–8	8	8	8	8
	e	8–10	11	11	10	10
No. 9	c	4–6	9	0	11	0
	d	6–8	4	0	8	0

than might have been expected. However, in the case of ovary only one of the 15 tested females produced a unisexual brood and then after an incubation period of 14–16 days. This low incidence and long incubation period from a tissue which clearly must carry the SR agent is remarkable and will be discussed below. On

TABLE 3

Tissues and organs tested for presence of the SR agent

Homogenates		SR.B-3—B-3 females				Control B-3—B-3 females			
Tissues and organs tested	Age and No. of flies	No. females injected	Progenies tested	No. SR	Incubation period days	No. injected	Progenies tested	No. SR	Incubation period days
Fat body	80 flies, 11 days	15	13	5	10–12	13	11	0	.....
Ovary	50 flies, 14 days	19	15	1	14–16	15	10	1	24–26
Thoracic muscle	50 flies, 20 days	14	9	3	16–18	13	7	1	22–24
Gut	80 flies, 11 days	12	2	0	.....	8	4	0	.....
Blood (from Table 1)	15 days	10	9	8	0–4	5	5	0	.....

the other hand the presence of SR agent in the thoracic musculature as shown by three SR progenies out of the nine tested females is somewhat unexpected, but also might be attributable to the high level of SR agent in the hemolymph in which it is bathed. The incubation period of 16–18 days is the longest in this series.

Among the controls, hemolymph, fat body, and gut proved negative. However, the occurrence of unisexual broods late in the lives of two females, one ovary-injected, the other muscle-injected, were most surprising findings. That these represented unequivocal instances of SR expression was proved by their persistence in subsequent generations. The very long incubation periods in these cases are in marked contrast to the more typical incubation periods encountered with tissues from the SR strain.

Tests of SR males: That the occasionally surviving sons of SR females do not transmit the SR condition to their progeny does not necessarily imply that these males are free of the agent, although this has been the assumption made in previous work with SR in *D. willistoni* (MALOGOLOWKIN, POULSON and WRIGHT 1959), as well as in *D. bifasciata* (MAGNI 1954) and *D. prosaltans* (CAVALCANTI, FALCÃO and CASTRO 1958). The high rate of infection from the hemolymph of SR females suggested tests of the occasional males in their progenies. The procedure used to test the blood of males was similar to that used above, except that, since only a single male was usually available at a given time and such males are often small and weak, it was necessary to make the tests within a day or two of the time of emergence. Further, in order to get any blood into the pipet from some of the males, it was necessary first to inject a small volume of Drosophila Ringer solution into the abdomen using the pipet to mix the Ringer solution with the hemolymph and subsequently withdrawing the mixture into the pipet for introduction into the recipient female. In any case the blood volume was sufficiently small that it served to inject only a single test female.

The SR males tested came from one of the lines of SR3g.R-3 and from one line of SR.B-3. In some instances the test females were from the B-3 strain, while in the other cases test females from the R-3 strain were used. The data on the progenies of the injected females (mated with males of their own strain) are presented in Table 4. Of the SR3g.R-3 injected B-3 females Nos. 1 and 3 gave SR infections

TABLE 4

Progenies from test females injected with blood from surviving sons of "sex-ratio" females. First two groups with blood from SR3g.R-3; lower group with blood from SR.B-3

Broods / Days	a 0–2		b 3–4		c 5–6		d 7–8		e 9–10		f 11–12		g 13–14		h 15–16		i 17–18		j 19–20		k 21–22		l 23–24		m 25–26		n–p 27–32		q–t 33–40	
B-3 ♀ ♀																														
1	0	0	4	7	19	22	23	7	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.	.
2	0	0	21	14	18	14	10	8	3	1	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3	0	0	0	0	17	16	15	13	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.	.
R-3 ♀ ♀																														
1	0	0	0	0	3	9	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
2	0	0	14	0	18	0	11	0	6	0	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3	0	0	4	0	8	0	10	0	9	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	.	.	.	.	.	.
4	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5	0	0	5	3	1	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B-3 ♀ ♀	Injection of $SR.B_3$ males blood into B-3 females																													
1	0	0	45	37	54	46	12	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	53	50	33	39	4	6	12	17	3	2	1	1	24	28	16	14	17	5	2	2	15	10	8	11	0	0	.	.
3	4	1	32	30	10	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5	0	0	0	0	0	0	0	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
6	0	0	19	13	1	5	1	0	0	0	0	0	0	0	0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	.
7	4	4	2	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.	.	.	.

which were confirmed by second generation tests. Although No. 2 showed fewer sons than normal the tested daughters from later broods gave no instances of SR. In the cases of the injected R-3 females Nos. 2, 3, and 4 gave completely SR broods (no males at all) and these were proved persistent through the second and several subsequent generations. Nos. 1 and 5 proved negative in second generation tests.

As summarized in Table 5 it is clear that the blood of five out of the eight tested SR3g.R-3 males carried SR agent. In the cases of males tested with B-3 females it was necessary to augment the blood volume with Drosophila Ringer solution as described above. The only apparent effect may be the increased incubation period of 6–8 days compared with a 2–4 day period for the R-3 recipients. While this might be interpreted as a host effect dilution is a much more probable explanation.

TABLE 5

Summary of tests of blood of surviving "sex-ratio" males for presence of SR agent. Test females injected with blood of SR males and then mated with males of their own strain

Donor males	Test females	No. females injected	No. SR progenies	No. females sterile	Incubation period	Mean survival time
SR3g.R-3*	B-3	3	2	0	6–8 days	25.3 days
SR3g.R-3	R-3	5	3	0	2–4 days	14.0 days
SR.B-3	B-3	7	0	2	..	25.1 days

* Blood diluted with small amount of Drosophila Ringer solution.

Fewer data are available for SR.B-3 males as they have usually been rare, and some injected females proved sterile in the lines under close observation. The results in each case were negative as shown in the lower part of Table 4. In view of the negative findings with hemolymph from normal B-3 and R-3 females no tests of blood of normal males from those strains have been made.

Tests of SR larvae: Observations on earlier developmental stages are somewhat limited. In view of the high level of SR agent in adult hemolymph examination of larval hemolymph was undertaken using the same procedures as above and third instar larvae of the two SR strains as sources of hemolymph. The results are presented in Table 6 where the major effect of the injection of

TABLE 6

Infectivity of larval blood of SR strains introduced into adult females of normal strains of D. willistoni

Donor strain	Test females	No. females injected	No. SR progenies	No. females sterile	Incubation period	Mean survival time
SR.B-3	B-3	10	0	7	...	21.4 days
SR3g.R-3	B-3	7	1	1	8–10 days	31.0 days
SR.B-3	R-3	8	0	5	...	16.3 days
SR3g.R-3	R-3	8	3	1	9–11 days	17.7 days

larval hemolymph from SR.B-3 is seen to be sterility of the recipient females. None of the six fertile recipients produced any SR progenies, but the sample is too small to draw the conclusion that the hemolymph of these larvae is free of SR agent. However, sterility was comparatively low when hemolymph from SR3g.R-3 larvae was introduced into females of either normal strain. One out of six fertile B-3 recipients produced SR progeny after an 8–10 day incubation period while three out of seven R-3 recipients gave SR after 9–11 days. These incubation periods are of the same order as found for SR abnormal male ooplasm (MALOGOLOWKIN, POULSON and WRIGHT 1959) and for adult supernatant (MALOGOLOWKIN, CARVALHO and DA PAZ 1960) and so suggest a comparable level of SR agent. As indicated in the right-hand column of the table the survival of the recipient females was markedly reduced as compared with those receiving adult SR hemolymph (Table 1).

DISCUSSION

The most important findings are the high level of SR agent in adult hemolymph, the contrasting relatively low level in the ovary, as well as the demonstration that the occasionally surviving males of the SR3g.R-3 strain carry the SR agent and, therefore, possess a considerable degree of resistance to its action. The high level of SR agent in adult blood raises the question of the extent to which this may be responsible for the apparent presence of the agent in the other tissues tested and, thus, whether the agent is primarily intracellular or extracellular in nature. Two things bear on this. First, the purely technical, or procedural, matter of the adequacy of washing of tissues to be tested. Insufficient washing could lead to apparent presence of the SR agent in tissues not actually containing it. This would be likely to happen with tissues such as fat body and muscle which are continuously bathed in hemolymph. Second, there is the situation in the controls where blood and fat body were negative in contrast to ovary and thoracic muscle. The definite but low level of SR agent demonstrated in the B-3 ovary and muscle make it perfectly clear that it is possible for these tissues, at least, to carry the agent when none is demonstrable in blood. While it is possible that the estimates of level of SR agent in the fat body and thoracic muscle of SR.B-3 may be affected by adherence of materials from the hemolymph this hardly appears to be the case for the ovary for which a lower, or at least comparable, frequency of infection was obtained in both strains. The longer incubation times for the two tissues in the normal B-3 strain are in keeping with expected lower levels in a non-SR as compared with an SR strain. While it might be considered that the number of tested females in this series was inadequate to demonstrate the existence of a low level of SR agent in the hemolymph of the B-3 strain, subsequent repetitions of hemolymph injection on a considerably large scale, as controls in other experiments (SAKAGUCHI and POULSON, unpublished), have consistently failed to give any evidence for the presence of SR agent in the blood of the B-3 strain. On the other hand another type of evidence for the presence of latent infection in the B-3 strain comes from the

finding (POULSON, unpublished) of a single female derived from the stock who produced only daughters and from whose progeny a new line of SR has been established. This female was the only one of many hundreds of tested B-3 females to show the condition. While it is possible that the B-3 stock became contaminated with SR earlier in its history in this laboratory a number of factors argue against such an interpretation (POULSON 1961). Thus whatever its origin the B-3 strain carries a latent, or attenuated, infection. To what extent this may be responsible for its properties as a maintainer strain for SR remains to be demonstrated, although evidence from other instances of attenuated infection are suggestive in this respect (SIMON 1960). Whether other strains are carriers of latent infections remains to be fully examined, although a further example of "spontaneous" appearance of SR has been found in the Feliz A wild stock (POULSON, unpublished). Clearly it would be difficult to prove that any given strain was completely free of SR agent. It is quite possible that the agent is widely distributed among wild populations of *D. willistoni*.

It is of some interest to consider the incubation times observed in this series of tests. These ranged from less than two days up to 26 days. Since it is quite clear from other work, especially that on CO_2-sensitivity (cf. review by L'HERITIER 1958), and our own studies on the effects of dilution of hemolymph POULSON and SAKAGUCHI 1961) that there is a close relation between incubation time and concentration of infectious agent, we can take incubation time as a measure of the relative concentration of the SR agent (unless inappropriate conditions or inhibitory substances be present). Thus the 24–26 day incubation period for the B-3 ovary represents a far lower level (about 1/50th) than that of the SR.B-3 ovary although the relative frequencies of infections were about the same. The relatively low level in the ovary (roughly 1/200) as compared with hemolymph in SR.B-3 females suggests that multiplication of the SR agent is, indeed, limited in the ovary, and this is borne out by the failure to obtain SR infections by injection of ooplasm of individual unfertilized eggs of SR females (POULSON, unpublished). Since the ovarian material was highly concentrated—100 ovaries in .03 ml—the above figures represent overestimates of the SR agent.

Relatively unrestricted multiplication of the SR agent in the hemolymph of adult SR females would appear to account for the high level encountered in flies of 10–20 days of age such as used here. Other data indicate that the level is much lower in the hemolymph of newly emerged flies, but not as low as found in third instar larvae. This high level in adult hemolymph led to a thorough investigation of the properties and nature of the SR agent reported in a preliminary way by us (POULSON and SAKAGUCHI 1960, 1961) and to be presented more fully in other papers of this series (SAKAGUCHI and POULSON, unpublished).

The presence of the SR agent in the occasional surviving SR3g.R-3 males invalidates the interpretation that they arise from zygotes lacking the agent, but poses the question of the mechanism of survival. Is it a combination of chance factors, the occurrence with low frequency of resistant male genotypes, or changes in the properties or type of the SR agent? Since only in the SR3g.R-3 strain was the hemolymph of surviving males found to be infectious and the

R-3 strain is known to be genotypically unfavorable (MALOGOLOWKIN 1958) to the persistence of the original SR condition, corresponding to what CAVALCANTI, FALCÃO and CASTRO (1958) call a disrupter strain, it seems pretty clear that the genotype of the flies is an important factor in male survival in the presence of SR agent. The problem warrants much more thorough study. Although the tests of hemolymph of surviving males of the SR.B-3 strain were negative it is not yet completely clear whether surviving males of the SR.B-3 strain carry the agent at a very low level or are free of it.

A comparison of the data of Table 1 with that of MALOGOLOWKIN, POULSON and WRIGHT (1959) shows that the performance of individual females was much more variable in the B-3 than in the R-3 strain, that the majority of progeny were produced from eggs laid during the first week, and that no adults developed from eggs laid after the 18th day. It is now clear from further studies that these features are characteristic of the B-3 stock. This early sterility was accentuated where hemolymph of SR.B-3 larvae was introduced into adult females (Table 6) of the B-3 strain and was produced in R-3 females receiving SR.B-3 larval hemolymph. A probable explanation for this peculiar reproductive behavior may lie in the fact (discovered after these experiments had been completed) that the B-3 strain regularly carries a microsporidian infection which is very persistent in mass cultures and is responsible for the failure of many single pair matings. By utilizing a variety of precautions and by careful selection it has since been possible to develop lines of B-3 and SR.B-3 free or relatively free of microsporidia. These are comparatively free of the sterility characterizing the original B-3 stock. A study of the characteristics and mode of transmission of the microsporidia is now under way in collaboration with DR. R. C. KING. The presence of the microsporidia in the B-3 strain presented a considerable complication in the early attempts to identify the SR agent. However, their absence in the Recife-3 and SR3g.R-3 strains made it possible to eliminate them from any direct relationship to the "sex-ratio" condition in *D. willistoni*.

SUMMARY

1. The infectious agent concerned in the production of the "sex-ratio" condition (SR) of unisexual progenies in *D. willistoni* was demonstrated through infection experiments to be present at an exceptionally high level in the hemolymph of mature adult females of two different SR strains. The SR agent is present in lower concentrations in fat body, thoracic muscle, and ovary. It is also present in hemolymph of third instar larvae.

2. The SR agent was found to be present at a low level in ovary and thoracic muscle, but lacking in the hemolymph and fat body, of adult females of the maintainer strain Barbados-3, thus providing evidence of latent or attenuated infection in that otherwise normal strain.

3. The occasionally surviving males of one SR strain (SR3g.R-3), maintained by mating to males of the Recife-3 strain, were found to possess a high level of

SR agent in the hemolymph. Tests of surviving males of the other strain (SR.B-3) were negative.

4. The bearings of the findings on the interpretation of maternally transmitted "sex-ratio" condition are briefly discussed.

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RECOMBINATION IN THE GENUS CUCURBITA

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Received August 8, 1961

VARIOUS structural and physiological barriers to recombination in species hybrids often impose rigorous limitations upon the extent to which the characters of one species may be combined with those of another. Among such barriers structural and genic imbalance resulting in the differential elimination of gametes and zygotes of recombination types has often been mentioned (ANDERSON 1939; DEMPSTER 1949; STEBBINS 1945; STEPHENS 1950). Others can be pleiotropy and linkage (ANDERSON 1939; DEMPSTER 1949); the multiple factor control of character differences between parental types (DEMPSTER 1949; SMITH 1950); developmental restrictions imposed by similar relative growth patterns (SMITH 1950); and affinity, a newly formulated theory which "proposes that centromeres of the same ancestral origin tend to travel to the same pole," (WALLACE 1960).

It was the objective of this investigation to determine the amount of recombination occurring in the species cross *C. pepo* × *C. moschata*, and to reveal the nature of restrictions to recombination between the two species.

MATERIALS AND METHODS

Two species of Cucurbita, a genus endemic to the Western Hemisphere, were chosen for this study: *C. moschata* Duch. (n=20), and *C. pepo* L. (n=20). Evidence gathered by many investigators shows the range of *C. moschata* to be from northern South America (Peru), through Central America, and into southern Mexico (BUKASOV 1930; CARTER 1945; WHITAKER 1960); and that of *C. pepo* from central Mexico, through northern Mexico, to Florida, and northward to Canada (WHITAKER and BOHN 1950; WHITAKER 1960). The latter species appears to have reached its greatest diversity along the Atlantic coast of eastern North America (WHITAKER and CARTER 1946). Neither of these species has ever been found in a truly wild state; rather they have always been associated with the human inhabitants in the areas of species distribution (WHITAKER and BOHN 1950; WHITAKER 1960).

The two cultivars chosen for this study were *C. pepo* var. Yankee Hybrid, a recent commercial introduction derived from Yellow Straightneck, which in turn had its origin in the eastern North American complex of *C. pepo* var. Melopepo Alef., and *C. moschata* var. Butternut, a commercial introduction of about 20

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years ago which WHITAKER (1960) considers to have been developed by simple selection from one of the Cushaw types. These varieties of the two species are morphologically dissimilar and genetically isolated. The accumulated evidence indicates that these species will not hybridize naturally (WHITAKER 1960). Artificial cross-pollinations are much more difficult to effect when *moschata* is the female parent than when it is the male parent. Fruits which set from this cross in either direction contain very few viable seeds or none at all. The details of the experimental methods have been presented previously (WALL and YORK 1960) and will not be repeated here.

Morphological differences: The overall differences between *C. moschata* and the bush form of *C. pepo* are considerable. There are no less than nine key character differences, though only a few of these can be scored objectively. After several trials it was decided to limit the characters used to internode length (as a measure of plant habit), sepal width, and androecium length. These three character differences are summarized in Table 1.

Measurement of fertility: Two methods were used to measure the fertility of the various populations. In the early stages of the study the fertility of the parental species and the F_1 hybrids was determined by counting the developed seeds present in all harvested fruits as compared with empty seeds. This method was also used in determining the frequency of success obtained in making the first backcross to the parental species.

As the size of the populations increased it became apparent that a more efficient method of measuring fertility was needed. Hence, it was decided to use the amount of pollen sterility for measuring the fertility of the F_2 and first backcross populations. Temporary mounts were made using ZIRKLE's (1937) mounting medium, in which moderate viscosity and jelling minimize the drift of abortive grains from under the cover slip. Pollen from freshly dehisced anthers was thoroughly mixed in a drop of medium, mounted, allowed to jell for a few moments, and then examined. Grains which were shriveled and unstained were classed as nonfunctional, and the well-filled, stained grains as functional. The sample size was 200 grains per slide with multiple counts being made on as many plants as possible. The accuracy of this method was found to be in close agreement with that of the zygote abortion method.

RESULTS

Recombination in the F_2: The analysis of the F_2 and first backcross generations are presented in scatter diagrams after the method of ANDERSON (1939). Figure 1

TABLE 1

Tabulation of character differences used in this investigation

	C. moschata	*C. pepo*
Internode length	5.8–8.5 cm	0.5–0.8 cm
Sepal width	somewhat expanded	narrow, awl-like
Androecium length	long, slender	short, conical

presents the recombination obtained in the F_2 population of 92 plants for sepal width with internode length. Figure 2 shows the recombination for 81 plants in the same F_2 for androecium length and internode length. These figures suggest that there is relatively unrestricted recombination of the genes conditioning internode length, sepal width and androecium length. In addition it is evident that the data are skewed toward the *pepo* phenotype, pronouncedly so for internode length, less so for sepal width and only faintly for androecium length. Table 2 presents the frequency distribution for F_2 for internode length, in which skewness toward *pepo* is readily apparent.

Recombination in the first backcross to parental species: Figure 3 compares the first backcross to *pepo* with the first backcross to *moschata* for the characters

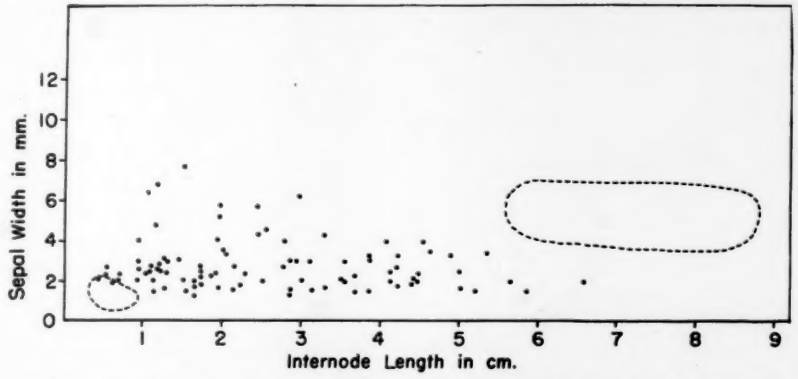

FIGURE 1.—Recombination of sepal width with internode length in the F_2 of 92 plants from *Cucurbita pepo* × *C. moschata*. The broken-line enclosures represent the range of the parental types.

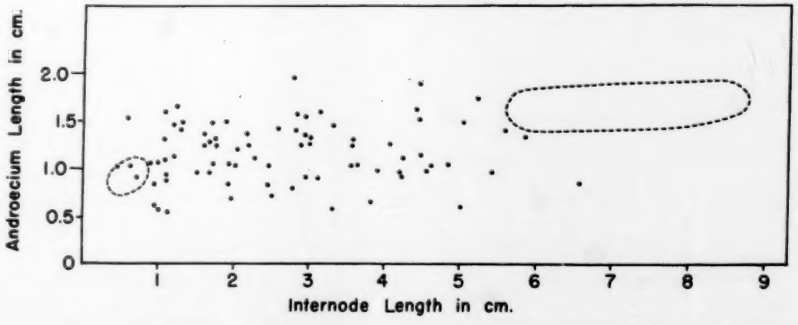

FIGURE 2.—Recombination of androecium length with internode length in the F_2 of 81 plants from *C. pepo* × *C. moschata*. The broken-line enclosures represent the range of the parental types.

TABLE 2

Frequency distribution for 92 plants of the F_2 of C. pepo × C. moschata *for mean internode length for five internodes beginning at base of plant*

	P_1	F_2					P_2
Length in cm	0.72	1	1–2	2–3	3–4	4–5	5	7.36
Number of individuals	...	10	32	17	13	13	7	...

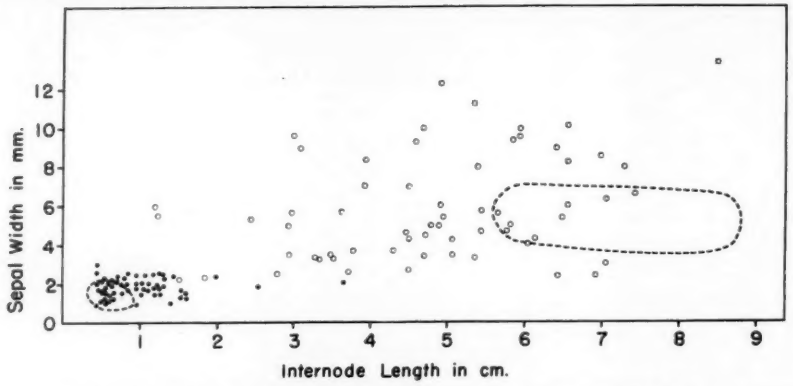

FIGURE 3.—Recombination of sepal width with internode length in the first backcross populations. Solid circles show the $F_1 \times C.$ *pepo* (58 plants); open circles show *C. moschata* × F_1 (62 plants). The broken-line enclosures represent the range of the parental types.

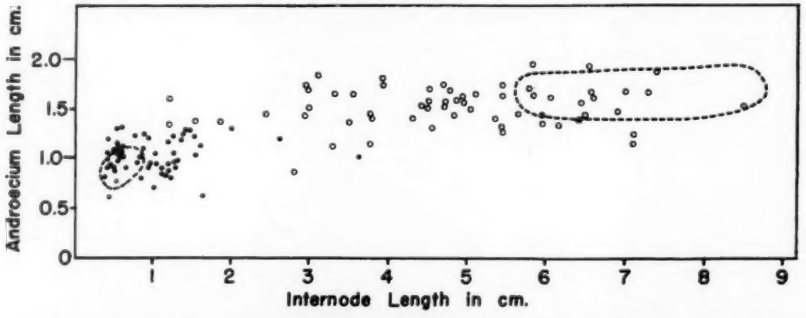

FIGURE 4.—Recombination of androecium length with internode length in the first backcross populations. Solid circles show the $F_1 \times C.$ *pepo* (56 plants); open circles show *C. moschata* × F_1 (62 plants). The broken-line enclosures represent the range of the parental types.

sepal width and internode length. In the backcross to *pepo* there is pronounced clustering of the progeny within and about the parental range. There is wide scattering of the progeny in the backcross to *moschata* and a number of transgressive segregants for sepal width were obtained. The same general relationship is true for these backcross populations for androecium length and internode

length (Figure 4), though there are fewer and less extreme transgressive types for androecium length than there were for sepal width.

Fertility relationships: The fertility relationships of the parental species, F_1, and F_2, are presented in Figure 5. The F_1 fertility was 71 percent, the F_2 50.2 percent, the backcross (*pepo* × *moschata*) ♀ × *pepo* ♂ 72.3 percent, and the backcross *moschata* ♀ × (*pepo* × *moschata*) ♂ 69.4 percent. The range in fertility was essentially the same in all three segregating generations, i.e., from zero to 93 percent. An interesting feature of the data in Figure 5 is that the pooled F_1 BC to *pepo* was 30.5 percent less successful in producing viable zygotes than was the pooled F_1 BC to *moschata*.

In the first backcross to *pepo* there is a significant difference in the numbers of viable zygotes produced when the F_1 is the pollen parent as opposed to when it is the female parent (Table 3). This finding is not unique in interspecific backcrosses and has been reported by other authors from either direct or indirect evidence. It is probable that the recombination type male gametes are at a greater disadvantage than are the corresponding female gametes. This interpretation is not difficult to visualize when we consider the long and intricate male gametophytic existence.

In like manner, the first backcross to *moschata* produces more viable zygotes

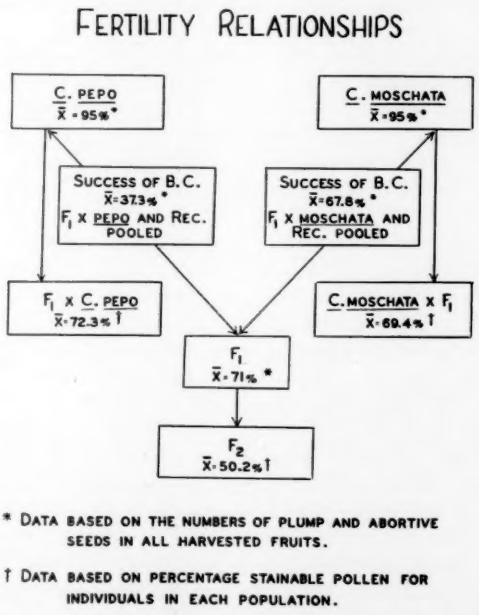
FERTILITY RELATIONSHIPS
C. PEPO
X̄ = 95%*
C. MOSCHATA
X̄ = 95%*
SUCCESS OF B.C.
X̄ = 37.3%*
F1 X PEPO AND REC. POOLED
SUCCESS OF B.C.
X̄ = 67.8%*
F1 X MOSCHATA AND REC. POOLED
F1 X C. PEPO
X̄ = 72.3%†
C. MOSCHATA X F1
X̄ = 69.4%†
F1
X̄ = 71%*
F2
X̄ = 50.2%†
* DATA BASED ON THE NUMBERS OF PLUMP AND ABORTIVE SEEDS IN ALL HARVESTED FRUITS.
† DATA BASED ON PERCENTAGE STAINABLE POLLEN FOR INDIVIDUALS IN EACH POPULATION.

FIGURE 5.—Schematic diagram depicting the fertility of the parental species, their F_1, F_2, and first backcross generation to each species. The percentage viable zygotes obtained when the F_1 was backcrossed to the parental species is also presented.

TABLE 3

The percentage of viable zygotes obtained when the F_1 was backcrossed to each parental species

	Females Males	Percent viable zygotes
First backcross to *pepo*	$F_1 \times$ *pepo*	44.7 ± 4.97
	pepo $\times F_1$	29.9 ± 2.28
First backcross to *moschata*	$F_1 \times$ *moschata*	68.6 ± 2.86
	moschata $\times F_1$	67.0 ± 2.96

when the F_1 is the seed parent than when it is the pollen parent, though the differences are not statistically significant.

DISCUSSION

Segregating generations: From the data it is seen that there are no serious restrictions to recombination in the F_2. The skewing of the F_2 data toward the *pepo* parent may be interpreted as an expression of partial dominance of *pepo* characters over *moschata* characters. *pepo* shows considerable dominance for internode length, less for sepal width and very little for androecium length. The backcross data are in general agreement with this interpretation. The backcross to *moschata* has a somewhat wider phenotypic range than would normally be expected and there is transgressive segregation for sepal width. However, this backcross is in *moschata* cytoplasm whereas the F_2 and first backcross to *pepo* are in *pepo* cytoplasm. There could be cytoplasmic effects in the backcross to *moschata* which to some degree may modify the expected phenotypic range.

No attempt has been made to determine the number of genes influencing each character under study. It should be noted, however, that what work has been reported on plant habit is not in full agreement. GREBENSCIKOV (1954) reported that in *C. pepo* plant habit is under multiple factor control with partial dominance of bush habit over vine habit. SHIFRISS (1947) reported that plant habit is determined by a single pair of alleles. A major feature of SHIFRISS' interpretation is that plants heterozygous for the plant habit locus are bush-like at the flowering stage but become vine-like at maturity. This situation he cites as an instance of developmental reversal of dominance.

When the F_1 was backcrossed to *pepo*, there was lower zygotic viability (37.3 percent) than in the F_1 selfed (71 percent). Why there should be such a great difference in frequency of zygotic abortion is not known, but it appears that in the backcross to *pepo* the more *moschata* genetic material a gamete contains, the more likely it is to not be fertilized or, if it is fertilized, to be aborted. This differential fertilization and/or zygotic abortion will result in less recombination than would otherwise be effected.

There was a considerable difference in the numbers of viable zygotes obtained when the F_1 was backcrossed to the two parental species (Table 3 and Figure 5). The pooled zygotic viability for the backcross to *pepo* was 37.3 percent and that for the backcross to *moschata* 67.8 percent. A highly significant difference. The

effect which this differential zygotic abortion has on the recombination types cannot be measured accurately in this work. It strongly suggests, however, that there is screening against the more extreme recombination types. It is not known why the backcross to *pepo* should result in greater zygotic inviability than the backcross to *moschata*, but genic-cytoplasmic interactions may be a major cause.

Fertility loss in F_2: The sharp decrease in fertility from the F_1 (71 percent) to the F_2 (50 percent) and the production of a number of unbalanced and sterile types in F_2, as occurred in this investigation, has its counterpart in species hybrids within other genera. HARLAND (1936) has reported the breakdown in interspecies crosses in Gossypium, viz., *G. barbadense* × *G. hirsutum* in the F_2, following the production of vigorous and fertile F_1 species hybrids. From his work on cotton he suggested an explanation for "interspecific breakdown" based on the theory of speciation through multiple gene substitution. He visualized that F_2 breakdown is the result of a rearrangement of the coadapted modifier systems contributed from the two species.

An equally plausible explanation of interspecific breakdown has been advanced based on small structural differences between homologous chromosomes in species hybrids. STEBBINS (1945) has suggested the term "cryptic structural hybridity" for these small, cytologically undetectable structural differences. STEPHENS (1950) has accumulated and assembled considerable data which support the thesis of cryptic structural differences between species and suggest that because of small structural differences between paired chromosomes there arise, through crossing over in the F_1, deficient gametic and zygotic types, "which would not necessarily be lethal or completely sterile." In cases of hybrid breakdown it is probable that the worst imbalance is screened out through gametic and zygotic abortion. In all likelihood interspecific breakdown occurs through a combination of multiple gene and cryptic structural differences, as STEPHENS (1950) has clearly indicated.

Exploitation of species differences: It is well to consider what method of mating will be most efficient in aiding gene transfer, or introgression, between these species. It was seen from the backcross data that pollen lethals are more effective in preventing gene exchange than are megaspore lethals. It follows from this finding that the F_1 should be used as the seed parent in bulk backcrossing to the recurrent parent. Pollen of the recurrent parent should be bulked for the backcrosses so as to adequately sample and maintain the genetic variability of the recurrent parent. Which parent is the female in the initial interspecific cross probably has an important bearing on the ultimate transfer of genetic material, since circumstantial evidence suggests that cytoplasmic differences do influence to some extent recombination in this species cross. But since the recombination and breeding data were obtained from only the one initial interspecific mating, *pepo* ♀ × *moschata* ♂, it is not definite which would be the better female parent.

It is suggested that the most useful way to exploit the interspecific differences between these species is to introgress one gene, or at the most a few genes, into the recurrent parent by backcrossing after the initial interspecific F_1 has been secured. Based on the results of this investigation, the progeny from this species

cross, either from controlled or random inbreeding, will slowly approach the parental species types with, finally, a complete loss of intermediate types. Introgression may ultimately result and be stabilized in true-breeding forms, but it will require a great number of generations. In a parallel case HUTCHINSON (1959) has estimated that it would take about 25 generations to obtain stable introgression types in the descendants of the species cross Upland cotton × Sea Island cotton (*Gossypium hirsutum* × *G. barbadense*) unless backcrossing is used. The same result might be achieved much more rapidly in Cucurbita via the backcross method, probably in as few as four or five generations.

SUMMARY

The extent of recombination occurring in the F_2 and first backcross generations of the interspecific cross *Cucurbita pepo* × *C. moschata* was investigated. It is concluded that relatively free recombination occurs in the F_2 for the genes determining internode length, sepal width and androecium length. The results of the first backcross generations in general support the conclusions obtained from F_2 data.

The first backcross to *C. pepo* was characterized by a sharp reduction in the number of viable zygotes obtained. While it cannot be stated with assurance that reduced viability is the result of gametic and zygotic abortion of the more extreme recombination types, it is highly probable. There was significantly greater zygotic viability in the corresponding backcross to *C. moschata*.

The fertility relationships of the F_1 and recombination generations suggest that a portion of the differences between these species are determined by chromosomal structural differences, as well as accumulated gene differences.

It is concluded that the most useful way to exploit the interspecific differences between these species is to introgress one gene, or at the most a few genes, into the recurrent parent after the initial interspecific F_1 has been secured.

ACKNOWLEDGMENTS

The author wishes to express his gratitude to DR. H. M. MUNGER and DR. S. G. STEPHENS for their helpful criticism of the manuscript.

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ANALYSIS OF PRODUCTS FROM REGULARLY OCCURRING ASYMMETRICAL EXCHANGE IN DROSOPHILA MELANOGASTER[1]

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Received August 25, 1961

THE regular occurrence of a deficiency product, apparently resulting from crossing over within an asymmetrically-paired segment of the *Drosophila melanogaster* X chromosome was reported by GREEN (1959). JUDD (1961) recovered the complementary duplication and deficiency strands from an asymmetrical exchange occurring within the white locus. Although the recombinant products analyzed by these two investigators are quite different, they are related since both demonstrate recombination in chromosome segments which are not specifically paired in a point-by-point manner. Prior to these investigations, asymmetrical crossover products were, in general, expected only from chromosomes bearing tandem duplications.

The regular recovery of duplication and deficiency recombinant products from structurally normal chromosomes allows several questions bearing on the mechanism of crossing over to be raised. First, since the exceptional types appear among the offspring from several different crosses, it is of interest to determine whether the non-homologous pairing is accomplished in precisely the same manner in each case, with subsequent crossing over always occurring at the same point. This would lead to a single type of duplication and the complementary deficiency. On the other hand, if pairing may occur in any one of several ways or if crossing over may take place at one of several places within the non-homologously paired region, duplication and deficiency products of several different types should be obtained. A related question is whether the asymmetrical pairing involves truly non-homologous chromosome regions or whether seemingly unrelated parts have retained some specific synaptic affinities during their evolution. In other words, how specific must pairing be in order to allow crossing over? Also, since the exceptional products are recovered from some crosses but not from others, a question is raised as to what genetic elements might be involved in determining this process.

Unlike the results reported by GREEN (1959), three different deficiency products and one duplication type have thus far been recovered from various crosses during the course of a study involving the spatial relationships between some white homoalleles and heteroalleles. A detailed genetic and cytological analysis

[1] This investigation was supported by PHS grant RG-6492 from the National Institutes of Health, Public Health Service.

of each exceptional recombinant has been carried out in an effort to determine its nature and mechanism of origin.

MATERIALS AND METHODS

A description of most of the mutants employed in this study may be found in BRIDGES and BREHME (1944). A list, along with symbols and map positions, is given in Table 1. Other special stocks are described in the text.

The flies were reared at 24°C on a standard Brewer's yeast, cornmeal, molasses, agar medium.

For the cytological analysis, larvae were reared at 20°C in uncrowded, yeast supplemented vials. Salivary glands were fixed in 1N HCl for 20 seconds, transferred to distilled water until swelling and clearing occurred and then transferred into a few drops of stain on a silicon-treated slide. The stain was two percent orcein in equal parts of glacial acetic acid and lactic acid (85%). A silicon-treated cover slip was placed over the glands and smearing was done by striking the cover slip sharply with a small rubber hammer. After blotting, the preparation was sealed with a mixture of paraffin and lanolin.

TABLE 1

A list of mutants used in this study. For more detailed descriptions, see BRIDGES *and* BREHME *1944*

Mutant symbol	Phenotype	Map position
y	yellow body and bristle color	X 0.0
y^2	yellow body; black bristles	X 0.0
sc	missing scutellar bristles (scute)	X 0.0+
Hw	Hairy wings	X 0.0+
z	zeste (yellow) eye color	X 1 ±
w	white eye color	X 1.5
w^{11E4}	white eye color	X 1.5
w^a	apricot eye color	X 1.5
w^{bf}	buff eye color	X 1.5
w^{Bwx}	brown eye color	X 1.5
spl	split bristles	X 3.0
N	Notch wings	X 3.0
dm	diminutive bristles	X 4.6
ec	echinus (rough) eyes	X 5.5
sn^3	singed bristles	X 21.0
lz^s	lozenge eyes	X 27.7
m^2	miniature wings	X 36.1
g^4	garnet eye color	X 44.4
f	forked bristles	X 56.7
SM1	Curly wings	II chromosome inversions
Ubx^{130}	Enlarged halteres	III chromosome inversions

RESULTS

Analysis of white-deficiency recombinants: Those crosses which yield white-deficiency recombinants (w^{-r}) are listed in Table 2. The parental female genotypes appear in the left-hand column; these females also carried *SM1*/+ and Ubx^{130}/+ in the second and third chromosomes in order to increase crossing over in the X chromosome. The male parents were *y w spl* sn^3 or *y w ec f* in genotype.

In the first two crosses listed, the w^{-r} products appear with regularity, while in the last three the number recovered is not large enough to support such a statement. The last cross, however, also yields a duplication type which appears to be the complementary strand of this w^{-r}; therefore, it is felt that this cross ($y^2 w^{r,dup}$ *ec*/+) along with the first two listed do give rise to asymmetrical exchange products with regularity. The w^a/w^{bf} and w^a/w^{Bwx} crosses are included here because the products recovered did without any doubt arise by crossing over, that is, in association with marker exchange between homologous chromosomes.

The w^{-r} females were originally recognized by their white phenotype and by the sex ratio of 2♀:1♂ among their offspring. Stock cultures were established from each exceptional female by balancing the deficiency against *In(1)*dl-49, bearing the markers *w* lz^s or *y Hw* m^2 g^4.

Each w^{-r} chromosome was subjected to a series of genetic tests as well as a careful cytological examination in order to characterize fully the change brought about by the recombination event. One of the genetic tests involved the use of three different white locus duplications compounded with each w^{-r}. If the duplication covered the deficiency, male w^{-r} offspring were obtained. Since the cytological limits of the duplications are known, coverage or failure to cover gives information concerning the limits of the deficiency in each w^{-r} type.

The duplications, Dp(1;2) w^{51b7} and Dp(1;4) w^{51c20}, described by RATTY (1954), extend over bands 3C2 to 3D2 and 3C2 to 3C6 respectively, using BRIDGES (1938) salivary gland chromosome map as the cytological reference. Dp(1;3) $w^{V,co}$ (BRIDGES and BREHME 1944) covers bands 2C1 to 3C4. The results from these tests are given in Table 3, and serve to separate the w^{-r} chromosomes into two groups. All of the w^{-r} types are covered by Dp $w^{V,co}$, with the exception of the one derived from w^a/w^{Bwx}, which in turn is covered by Dp w^{51b7}; Dp w^{51c20} fails to cover any of the w^{-r} types. This means that w^{-r} from w^a/w^{Bwx} can be de-

TABLE 2

The occurrence of exceptional recombinant females. All parental females were heterozygous for SM1 *and* Ubx^{130}. *Male parents were* y w spl sn^3 *or* y w ec f

Parental female genotype	Exceptional females	Total progeny
y^2 w^a/w^a *spl ec*	4 y^2 w^{-r} *spl ec*	49,884
y^2 w^a *spl ec*/+	6 w^{-r} *spl ec*	65,232
y^2 w^a *spl ec*/w^{bf} f^s	1 y^2 w^{-r} f^{s*}	63,001
y^2 w^a *spl ec*/*y z* w^{Bwx}	1 y^2 w^{-r} *N*	38,021
y^2 $w^{r,dup}$ *ec*/+	2 w^{-r} *ec*	34,085

* For other recombinant exceptions from this cross, see JUDD 1959.

ficient at a maximum for 3C2 to 3D2. This deficiency must extend through 3C7 to at least 3C10 since it exhibits a Notch phenotype and uncovers the recessive mutant *dm*. Diminutive is placed at 3C9 by SCHULTZ and at 3D1, 2 by DEMEREC (see BRIDGES and BREHME 1944). All the remaining types must be deficient for some part of the region extending from 2C1 to 3C4.

Additional information bearing on the limits of the w^{-r} types has been obtained by compounding them *inter se*, as well as with a series of known deficiencies. The assumption made in this test is that lethality will result if both chromosomes lack a common region. If the two deficiencies do not overlap, females will survive despite the fact that both of their X chromosomes would be lethal in the homozygous or hemizygous state. The deficiencies used as tester chromosomes were: w^{258-11}, deficient for bands 3A3 to 3C3 inclusive; a chromosome derived by crossing over between $In(1)rst^3$ and $In(1)w^{m4}$ which is deficient for bands 3C2, 3; w^{258-45}, deficient for band 3C1.

The results from these combinations are given in Table 4, and again serve to divide the w^{-r} chromosomes into the same two groups distinguished by the previ-

TABLE 3

Tests for coverage of w^{-r} *chromosomes by three white locus duplications. The cytological limits of each duplication are placed in parentheses.* + = *coverage by duplication;* 0 = *failure of coverage*

w^{-r} chromosomes	Dp(1;2)w^{51b7} (3C2–3D2)	Dp(1;4)w^{51cz6} (3C2–3C6)	Dp(1;3)$w^{V,co}$ (2C1–3C4)
y^2 w^{-r} *spl ec* (from w^a/w^a)	0	0	+
w^{-r} *spl ec* (from $w^a/+$)	0	0	+
y^2 w^{-r} f^s (from w^a/w^{bf})	0	0	+
y^2 w^{-r} *N* (from w^a/w^{Bwx})	+	0	0
w^{-r} *ec* (from $w^{r,\,dup}/+$)	0	0	+

TABLE 4

Tests of w^{-r} *chromosomes* inter se, *and with three white region deficiencies of known size.* L = *lethal combination;* w = *viable white-eyed females*

	w^{258-45} (3C1)	w^{258-11} (2C1–3C4)	w^{-rst^3*} (3C2, 3)	y^2 w^{-r} *spl ec* (w^a/w^a)	w^{-r} *spl ec* ($w^a/+$)	y^2 w^{-r} f^s (w^a/w^{bf})	y^2 w^{-r} *N* (w^a/w^{Bwx})	w^{-r} *ec* ($w^{r,\,dup}/+$)
w^{258-45}	L	L	w	L	L	L	w	L
w^{258-11}	..	L	..	L	L	L	..	L
w^{-} rst^{3*}	..	..	L	w	w	w	L	w
y^2 w^{-r} *spl ec* (w^a/w^a)	..	..	..	L	L	L	w	L
w^{-r} *spl ec* ($w^a/+$)	..	..	..	..	L	L	w	L
y^2 w^{-r} f^s (w^a/w^{bf})	..	..	..	..	..	L	w	L
y^2 w^{-r} *N* (w^a/w^{Bwx})	..	..	..	..	..	..	L	w
w^{-r} *ec* ($w^{-r,\,dup}/+$)	..	..	..	..	..	..	..	L

* Derived by crossing over between $In(1)w^{m4}$ and $In(1)rst^3$.

ously described use of the white duplications. Stricter limits may now be placed on the break points of some of the w^{-r} types, however. All of the w^{-r} losses with the exception of the type obtained from w^a/w^{Bwz} have one feature in common, that is the loss of band 3C1. The w^{-r} from w^a/w^{Bwz} on the other hand lives when compounded with a known 3C1 deficiency or with any of the other w^{-r} types. Interestingly enough, these combinations produce viable females which are white in phenotype.

The information gathered thus far allows the following conclusions to be drawn: (1) The w^{-r} from w^a/w^a, $w^a/+$, w^a/w^{bf} and $w^{r,dup}/+$ all have their rightmost break between bands 3C1 and 3C2, and extend left from that point, but not beyond 2C1. (2) The deficiency derived from w^a/w^{Bwz} has its leftmost break between 3C1 and 3C2 and extends to the right to some point between 3C9 and 3D2.

To delimit the extent of the deficiencies more closely, one other test was employed. Among the group with the missing region extending left from 3C1, it is of interest to determine whether the locus of zeste, located in band 3A3 (GANS 1953), is or is not included in the deficiency. To test for the presence of the zeste locus is not a simple operation since its mutant expression depends on the presence of two normal white regions. It was discovered, however, that an intralocus duplication for part of the white locus derived by crossing over between $w^{r,dup}$ (JUDD 1961) and a wild-type X chromosome, would serve to differentiate between those chromosomes having z^+ and those lacking z^+. The $w^{r,dup}$ chromosome was originally recovered from *y sc z* w^a *ec*/y^2 w^{bf} *spl* attached-X chromosomes and represents an intralocus duplication for white. The mutant w^{bf} is present in $w^{r,dup}$ and accounts in part for the "light buff" phenotype. The replacement of w^{bf} with a wild-type allele by crossing over results in a chromosome which still carries the intralocus duplication but which phenotypically is wild type. This crossover product is designated as $w^{r,dup+}$. Cytologically both $w^{r,dup}$ and $w^{r,dup+}$ appear normal; both, however, interact with the mutant *z*. It is this interaction with the zeste locus that allows the test for presence or absence of z^+.

To demonstrate the validity of the test, two of the white deficiencies employed earlier were used. w^{258-11}, which is deficient for 3A3 to 3C3 inclusive, lacks the *z* locus, while w^{258-45} is deficient for 3C1 only and thus has z^+ present. When these two deficiencies were compounded with *z* $w^{r,dup+}$, the class of genotype w^{258-11}/*z* $w^{r,dup+}$ was zeste in phenotype while w^{258-45}/*z* $w^{r,dup+}$ females were a dark reddish-brown. As a further check on this test, flies of genotypes *z* w^+/*z* $w^{r,dup+}$ and *z* w^{11E4}/*z* $w^{r,dup+}$ were obtained and both proved to be zeste in phenotype, while z^+ w^+/*z* $w^{r,dup+}$ and z^+ *w*/*z* $w^{r,dup+}$ were a dark reddish-brown mottle in eye color. Both *w* and w^{11E4} are suppressors of *z*, just as is a deficiency for the white locus (GANS 1953). It is reasonable to assume then that this test does distinguish between the presence or absence of z^+ regardless of whether the deficiency extends through the white locus.

This same test was performed with each of the w^{-r} chromosomes and served to further subdivide the classes. The w^{-r} chromosomes obtained from w^a/w^a lack

the zeste locus while those from $w^a/+$, w^a/w^{bf}, $w^{r,dup}/+$ all have it present, as of course does w^{-r} derived from w^a/w^{Bwz}.

Taking into consideration all of the genetic tests, three categories may be established among the w^{-r} chromosomes. (1) The w^{-r} from w^a/w^a is deficient for 3A3 to 3C1 inclusive. The left break is not established with certainty by these tests but must lie between 3A3 and 2C1. (2) w^{-r} chromosomes derived from $w^a/+$, w^a/w^{bf} and $w^{r,dup}/+$ have the deficiency extending from 3A4 to 3C1 as a maximum. Again, the left break is not definitely established by the genetic tests, but it is clearly different from that of w^{-r} recovered from w^a/w^a. (3) The w^{-r} from w^a/w^{Bwz} is deficient for 3C2 to 3D2, with the right break not established with certainty.

It should be noted that all three types of recombinant chromosomes have one break in common, that is, between 3C1 and 3C2; also, that any given cross yields but a single type of deficiency. With these results in mind, it was decided to do a detailed cytological study of each of the exceptional chromosomes in order to more accurately define the break points.

Cytological analysis of white-deficiency recombinants: The cytological studies served to confirm the categories established through the genetic tests and allowed a more definite establishment of break points. Despite a very careful analysis, no differences were found among the deficiencies recovered from any given cross. Utilizing the data collected from both the genetic and cytological studies, the limits of the three different w^{-r} types may now be stated as follows:

w^{-r} from w^a/w^a extends from 3A3 to 3C1 inclusive;

w^{-r} from $w^a/+$, w^a/w^{bf} and $w^{r,dup}/+$ extends from 3A8 to 3C1, with the left break somewhat uncertain due to the very faint bands present in this area;

w^{-r} from w^a/w^{Bwz} extends from 3C2 to 3C12, with 3D1,2 uncertain.

Photomicrographs of representatives of each of the three w^{-r} types are presented in Plate 1. Although exact break points cannot be determined from the photographs, they are sufficient to illustrate that the limits of each deficient type are unquestionably different.

Analysis of recombinant duplication: In only one cross, other than the one devised by JUDD (1961) which allows the recovery of the small intralocus white duplication, has it been possible to recover a recombinant chromosome which represents the duplication resulting from asymmetrical exchange. Females of genotype $y^2\ w^{r,dup}\ ec/+$; $SM1/+$; $Ubx^{130}/+$ when mated to $z\ w^{11E4}$ males yield a product, carrying the marker y^2, which is recognized in the female offspring of this cross by its very dark brown, slightly mottled phenotype. Five of these exceptions were recovered among 74,003 offspring. Progeny testing of these exceptional females showed that the recombinant chromosome (designated here as $w^{r,Dp}$) exhibited a wild-type eye color and is male viable. The white duplication, however, interacts with the mutant z to account for the dark brown mottled phenotype of the F_1 female exceptions. It should be noted that the $y^2\ w^{r,Dp}$ chromosome appears to be the reciprocal product of the $w^{-r}\ ec$ derived from the same type of parental females, the analysis of which is reported in the preceding section. Cytological analysis of the five $w^{r,Dp}$ chromosomes shows a duplication

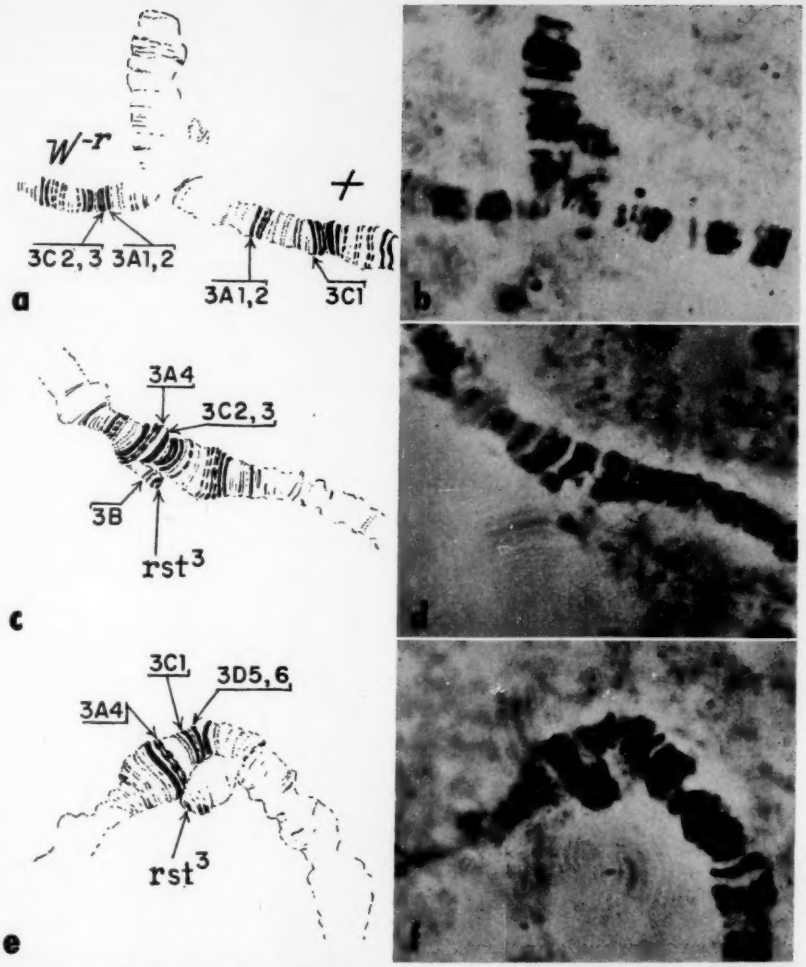

PLATE 1.—Photomicrographs and drawings of three types of white-deficiency recombinants. Sections a and b show w^{-r} from w^a/w^a heterozygous for a structurally normal X chromosome. The two strands are separated from one another with the + strand extending to the right and w^{-r} to the left. Note the absence of bands 3A3 through 3C1 in w^{-r}. Sections c and d show w^{-r} from $w^a/+$ paired with a rst^3 chromosome. Note the absence of region 3A8 through 3C1. Sections e and f show the $w^{-r}N$ chromosome from w^a/w^{Bwz} paired with rst^3. Bands 3C2 through 3C12 are missing in the w^{-r} strand.

which includes 3B1 through 3C1 as a minimum. This corresponds rather closely to the section which appears to be missing in the presumed reciprocal w^{-r} chromosome. Plate 2 is a photomicrograph of one of the $w^{r,Dp}$ chromosomes.

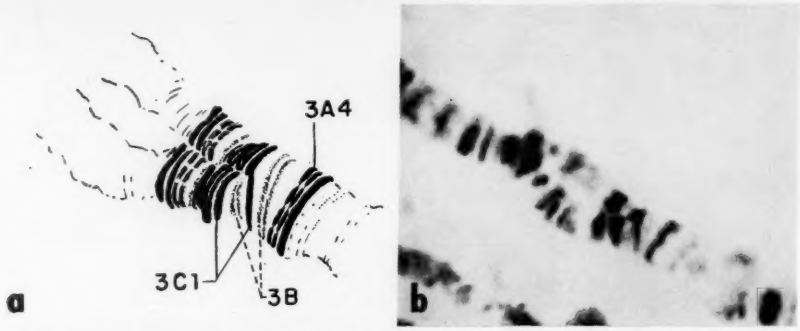

PLATE 2.—Photomicrograph and drawing of $w^{r,\,Dp}$ from $w^{r,\,dup}/+$ paired with a structurally normal chromosome. Note the duplication which extends from region 3B through 3C1 as a minimum.

The cytological limits of $w^{r,Dp}$ are similar to those reported by LEWIS (1957) for Dp(1;1) w^{55j}, which appeared as a *y ac* (w^{ch}) (*sp-w*) son of a *y ac* w^{ch} *fa*/y^{2} *sp-w* female. The origin of Dp(1;1) w^{55j} could be through asymmetrical exchange similar to the cases described here. The genetic analysis by LEWIS indicated, however, that the locus of roughest is also included in the duplicated segment, therefore, it is probably not identical with $w^{r,Dp}$.

A complete analysis of the $w^{r,dup}$ chromosome from which these more extensive duplication and deficiency recombinant products were derived is presently underway and will be reported at a later time. As has already been reported (JUDD 1961), this product, $w^{r,dup}$, appears to be structurally normal even though a portion of the white locus is duplicated. At this point in the investigation, it appears that this intralocus duplication is having little if any effect on the type of asymmetrical pairing reported here and by GREEN (1959). It is significant that the cross involving the intralocus duplication was the only one which allowed the recognition of the larger $w^{r,Dp}$ exception. This is attributable to the use of the mutant *z* in the male parent of this cross, and one would predict that the reciprocal duplications might be obtained from all of the crosses reported here if the mutant *z* is used as a detection device. It is possible of course that not all of the duplication types will give this interaction with zeste and some other indicator system must be used to recover them.

DISCUSSION

Several interesting facts emerge from this study of recombination products. It is considered significant that only one type of asymmetrical exchange is detected from any given cross, despite the demonstration that exchanges at other points in this region can be recognized. This observation can best be explained by assuming that the non-homologous pairing always occurs in the same way in any given genotype; and furthermore, that exchange between homologs paired

asymmetrically may occur only within some very restricted area. The points of pairing and exchange appear to be different for different genotypes.

GREEN (1959) postulates a pairing scheme which when accompanied by crossing over would yield the w^{-r} type which he describes. Such a scheme involves the pairing of band 3A3 (the *z* locus) with band 3C1 of the homologous chromosome. A similar but not identical pairing arrangement can be used to explain the w^{-r} obtained from w^a/w^a. In this case band 3A3 would pair with band 3C2 of the homolog in the following way:

$$\begin{array}{l} \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad \ldots 3A1,2,3,4 \ldots 10\ 3B1,2\ \ 3C1,2,3,4 \ldots \\ 3A1,2 \ldots 3B1,2 \ldots 3C1,2,3 \ldots\ldots\ldots 12\ \ 3D1,2,3,4 \ldots \end{array}$$

A crossover occurring between 3A2,3 in the upper sequence and between 3C1,2 in the lower would give a chromosome deficient for 3A3 to 3C1 inclusive and the other strand would contain a tandem duplication for this region. All of the known white locus mutations appear to be located in the 3C2,3 doublet which under this scheme is pairing with the closely linked zeste locus (also an eye-color determining locus) at 3A3. This might be taken as evidence that the white and zeste loci evolved from a common ancestral unit and that some pairing homology still remains. Additional evidence for this view comes from the functional relationships which exist between these two loci (GANS 1953).

The pairing scheme above brings the 3C2 band in the upper sequence adjacent to 3D3 in the lower sequence if a band for band pairing along the length of this chromosome segment is assumed. A crossover between 3C1,2 and 3D2,3 would give a chromosome deficient for 3C2 to 3D2 inclusive. These limits correspond rather closely to those of the w^{-r} derived from w^a/w^{Bwz}.

In order to explain the exceptional recombinants from $w^a/+$, w^a/w^{bf} and $w^{r,dup}/+$, a pairing arrangement quite different from the one just described must be assumed. In this case, the deficiency extends from about 3A8 to 3C1. The pairing which would most simply give this type of crossover product is as follows:

$$\begin{array}{l} \quad\quad\quad\quad \ldots 3A1,2 \ldots 6,7,8,9,10\ \ 3B1,2 \ldots 3C1,2 \ldots \\ 3A1,2,3 \ldots 3B1 \ldots 3C1,2,3 \ldots . \end{array}$$

A crossover between 3A7,8 in the upper sequence and between 3C1,2 in the lower would give duplication and efficiency products with break points at 3A8 and 3C1. It is of course quite likely that the salivary gland chromosome bands do not accurately portray the chromosome region as it is at the time crossing over occurs. Nonetheless, it is difficult to reconcile the origin of all of the w^{-r} chromosomes with a single asymmetrical pairing scheme, unless one assumes that this chromosomal region is lengthened at prophase by a genetically inert substance such as intercalary heterochromatin. PROKOFYEVA-BELGOVSKAYA (1939) notes the possibility of heterochromatin in the 3C region, based on the cytological observation that inert regions tend to pair with one another. Under these conditions, exchanges which occur within some relatively extensive region would all appear to have taken place at the same point genetically and on the salivary gland chromosome. Such a scheme would tend to group the exchanges

at a few sites. This still would not explain why all of the exceptions from any single cross appear to be identical however.

GREEN (1959) attempted to evaluate the genetic factors involved in the determination of the occurrence of w^{-r} chromosomes and came to the conclusion that the mutant apricot or a related mutant was necessary for the origin of the w^{-r} losses. It should be pointed out that all but one of the crosses used in this present study involved the mutant apricot. More than 20 other crosses, some also using apricot, failed to give w^{-r} types.

GREEN also states that the genetic state of the *z* locus, at least in the w^{a2} chromosome he used, plays an important role in the mechanism of origin of w^{-r} chromosomes. This point also can be supported by the present study by citing the following example. Females of genotype y^2 w^a *spl ec*/w^{Bwz} yield the reciprocal crossover types expected from heteroalleles, i.e., y^2w^+ and w^{Bwz} w^a *spl ec*, and in addition a y^2 $w^{r,def}$ class (JUDD 1959) which represents an intralocus deficiency similar to that reported by JUDD (1961). Presumably the reciprocal intralocus duplication class also occurs but was not recognized. If, however, the mutant *z* is placed in the w^{Bwz} chromosome and females of genotype y^2 w^a *spl ec*/*y z* w^{Bwz} are constructed, the intralocus deficiency (y^2 $w^{r,def}$) class is not recovered; instead as reported here, a y^2 w^{-r} *N* type deficient for 3C2 to 3C12 is found. Presumably the duplication product reciprocal to the w^{-r} also occurs but goes undetected because of the way the cross was performed. The substitution of the mutant *z* into the w^{Bwz} chromosome influences the type of asymmetrical pairing in a very dramatic way. This points once more to genetic control of pairing and crossing over, and implies that even asymmetrical pairing is still quite specific.

It is not possible at this point to determine which if any of the w^{-r} types described here corresponds to the one reported by GREEN (1959). His analysis showed that the z^+ locus was present in the w^{-r} chromosome; therefore, it is similar to the type which is deficient for 3A8 to 3C1, obtained from $w^a/+$, w^a/w^{bf} and $w^{r,dup}/+$. Since GREEN did not carry out a cytological examination, it remains to be determined whether these two types are identical.

The regular and relatively frequent occurrence of rather extensive duplication and deficiency products by crossing over provide changes in chromosome structure which are of considerable significance to the evolution of this organism. The deficiencies obviously will be selected against and rather rapidly eliminated. The duplications on the other hand, if we may judge by the type which has been recognized, are completely viable and fertile and for the most part not recognizably different from their normal sibs. The presence of a duplication, however, allows sites for the occurrence of new mutations without doing away with gene functions which might be indispensable. Such duplications could add immeasurably to the variability within a population without seriously affecting that part of the genome which is already selectively adapted. Evolutionary changes in such a population could be more rapid, and the types of mutations tested by natural selection could be larger in number and more varied in type.

It is considered quite plausible that the recombination events described here

will be found in a variety of organisms and will prove to be an important factor in evolution.

SUMMARY

Three types of white deficiencies and one type of white locus duplication which apparently arise by crossing over within an asymmetrically paired chromosome region are described by genetic tests and from cytological observations. Only a single type of asymmetric exchange occurs in any given cross.

Pairing schemes are presented which will allow the formation of the exceptional recombinants. It is concluded that the asymmetric pairing is genotypically controlled and is quite specific.

ACKNOWLEDGMENTS

The excellent technical assistance by Miss Carolyn Luerson and later by Mrs. Lynn Chertkov is gratefully acknowledged.

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THE MAINTENANCE OF SUPERNUMERARY CHROMOSOMES IN WILD POPULATIONS OF LILIUM CALLOSUM BY PREFERENTIAL SEGREGATION[1]

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Received August 25, 1961

A CONSIDERABLE number of plant species have been reported to contain supernumerary or accessory chromosomes (B chromosomes) in some members of their population, and the list of such species is steadily increasing. Recently, MÜNTZING and his collaborators (MÜNTZING 1954) have carried out extensive cytogenetical investigations of supernumerary chromosomes in Secale, Festuca, Centaurea, Poa and Anthoxanthum. One of the most interesting points involved is that in such a case as in rye, the total effect of B chromosomes is predominantly deleterious but at the same time there is a special mechanism which effects a numerical increase of the B chromosomes in the functional male gametes, thus leading to a balance between two opposing forces. A similar result has been reported by the junior author (KAYANO 1956b, 1957) in *Lilium callosum*, in which a supernumerary chromosome denoted by f_1 (meaning long telocentric fragment) has a tendency to increase in numbers due to preferential segregation in embryo-sac-mother cells. Thus by crossing a plant with one f_1 chromosome as a seed parent and one with no f_1 chromosome as a pollen parent, he obtained plants with one f_1 and no f_1 in an approximate ratio of 80:20. This agreed well with the cytological observation that in about 80 percent of the cases f_1 goes to the micropylar side to be included in the egg cell. Pollen fertility and seed setting have also been investigated in plants with various numbers of f_1 chromosomes. As will be shown in the next section, f_1 chromosomes depress the reproductive ability of the possessor rather severely so that plants with more than two f_1 chromosomes are almost completely sterile. In order to investigate the distribution of the supernumerary chromosome among individuals of wild populations, samples were collected from several localities and examined cytologically. The pattern of distribution was quite clear, and it was realized by the senior author that with observational data on pollen fertility and seed setting it might be possible to analyse the mechanism of distribution of the f_1 chromosome by developing a mathematical model. In the present paper, the junior author is responsible for experimental and observational data, while the senior author is responsible for mathematical and statistical analyses.

[1] Contribution No. 390 from the National Institute of Genetics, Mishima-shi, Japan. Aided in part by a Grant-in-Aid for Fundamental Scientific Research from the Ministry of Education, Japan, No. 0030.

Experimental and observational data

Samples were collected from four colonies or populations denoted by I, II, III and IV.

Populations I, II and III are located on a calcareous terrain of Hirao-Dai near Kokura-shi, Fukuoka Prefecture and they are about two kilometers apart. Population IV was found on a slope in the suburbs of Karatsu-shi, Saga Prefecture and is quite far from the other three populations. In this area the soil is not calcareous. In all these places, *L. callosum* was found growing together with *Miscanthus sinensis*.

In Figures 1 a–d, the results of karyotype analysis are presented together with maps showing the spatial distribution of individuals within each population. It may be seen from these maps that the distribution of supernumerary chromosomes is quite striking, one type denoted by f_1 being found in a large majority of plants and yet its number per individual being restricted to one or two (for the description of the types of supernumerary chromosomes, see KAYANO 1956a). Frequency distributions of the number of f_1 chromosomes within the four samples are summarized in Table 1. Pollen and seed fertilities were investigated using individuals from population I and the results are summarized in Tables 2 and 3. These figures represent averages over two years in the case of pollen fertility (1953 and 1954) and over three years for seed fertility (1952, 1953 and 1954).

The characteristic mode of transmission of the f_1 chromosome has already been reported by the junior author (KAYANO 1956b), but it may be convenient to

TABLE 1

Frequency distribution of the number of f_1 chromosomes

Populations sampled No. of f_1	I (percent)	II (percent)	III (percent)	IV (percent)	Sum (percent)
0	17 (28.8)	10 (18.5)	18 (39.1)	29 (46.0)	74 (33.3)
1	30* (50.9)	33‡ (61.1)	26 (56.5)	31 (49.2)	120 (54.1)
2	12† (20.3)	11 (20.4)	2 (4.3)	3 (4.8)	28 (12.6)
Total no. of individuals observed	59 (100)	54 (100)	46 (100)	63 (100)	222 (100)

* Contains two individuals having $1f_1 + 1f_s$, and one individual having $1f_1 + 1F_1$. (f_s means a short supernumerary fragment).
† Contains one individual with $2f_1 + 1f_s$.
‡ Contains one individual with $1f_1 + 3f_s$.

TABLE 2

Pollen fertility of individuals with various numbers of f_1 chromosomes

No. of f_1	No. of plants investigated	No. of fertile pollen grains	No. of sterile pollen grains	Total no. counted	Fertility (percent)
0	24	17,621	879	18,500	95.25
1	38	26,739	3,761	30,500	87.67
2	21	8,521	7,479	16,000	53.26

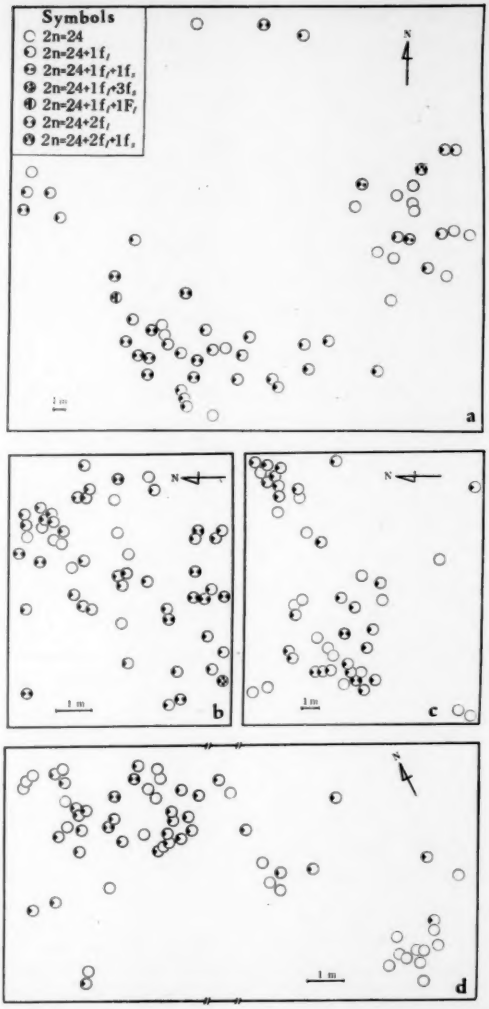

FIGURE 1a–d.—Spatial distribution of individuals with various number of f_1 chromosomes within four colonies of *Lilium callosum*. a, b and c (populations I, II and III) were located on a calcareous terrain of Hirao-Dai near Kokura-shi, Fukuoka Prefecture; these are about two kilometers apart. d (population IV) was found on a slope in the suburbs of Karatsu-shi, Saga Prefecture; it is quite far apart from the others.

reproduce the experimental results in the form of Table 4, in which the figures given in parentheses are the approximate values used as bases for calculation.

A crossing experiment conducted in 1958 revealed that this lily was self-incom-

patible. The results of this experiment are listed in Table 5. Thus *L. callosum* may be regarded as an outbreeding species. Furthermore, it reproduces, at least in part, asexually through development of new bulbs.

Mathematical and statistical analyses

1. *Mathematical model:* In order to analyse the present case, we must take into account two new features which do not appear in the ordinary mathematical treatment in population genetics. Namely, (a) both sexual and asexual reproduction have to be considered and (b) the mode of segregation of the f_1 chromosome does not follow the simple rules characteristic of ordinary diploid loci. With these two points in mind, we will formulate the problem as follows, in which an abbreviated symbol f will often be used to represent the f_1 chromosome.

TABLE 3

Seed fertility of individuals with various numbers of f_1 chromosomes

No. of f_1	No. of individuals investigated	No. of fertile seeds	No. of sterile seeds	Total no. of seeds counted	Fertility (percent)
0	15	4,662	2,907	7,569	61.59
1	25	9,102	8,305	17,407	52.29
2	12	3,228	5,859	9,087	35.52

TABLE 4

Mode of transmission of f_1 chromosome in artificial crosses

No. of f_1 in the offspring No. of f_1 in the parents ♀ × ♂	0	1	2	3	No. of offspring observed
0 × 1	45(50)	54(50)	..	..	99*
1 × 0	16(20)	83(80)	1	..	100
1 × 1	8(10)	53(50)	38(40)	1	100
0 × 2	7	88	5	..	100
2 × 0	5	76	18	1	100

* One individual having chromosome $2n=24+1F_1$ is excluded.

TABLE 5

Comparison of the effects of outcrossing and selfing on the development of capsules

	No. of flowers pollinated	No. of capsules developed	No. of capsules failed to develop
Outcrossing			
Between individuals with no f_1	11	10	1
Between individuals with 1 f_1	21	19	2
Between individuals with different karyotypes	17	17	0
Total	49	46	3
Selfing	20	(2)*	18

* Development was incomplete and did not contain fertile seeds.

Let Φ_i be the relative frequency of mature individuals with i f-chromosomes ($i = 0, 1, 2, \ldots$) in the population, and let w_i^* and w_i^{**} be the relative fertilities or reproductive values of individuals with i f's as pollen and seed parent respectively. We will designate by $g_{j/i}^*$ the relative proportion of pollen with j f-chromosomes among fertile pollen produced by individuals having i f-chromosomes, and by $g_{j/i}^{**}$ the proportion of fertile egg cells with j f-chromosomes produced by the same individual. Then we can write the male gametic array giving the frequency distribution of the number of f chromosomes in the male gametes which contribute to the next generation as

$$(1) \qquad \sum_j G_j^* f^j \equiv G_0^* + G_1^* f + G_2^* ff + \ldots + G_j^* f^j + \ldots,$$

in which

$$G_j^* = \frac{\sum_i g_{j/i}^* w_i^* \Phi_i}{\sum_i w_i^* \Phi_i} \qquad (j = 0, 1, 2, \ldots)$$

is the proportion of pollen with j f's. Similarly the female gametic array may be written in the form:

$$(2) \qquad \sum_k G_k^{**} f^k \equiv G_0^{**} + G_1^{**} f + G_2^{**} ff + \ldots + G_k^{**} f^k + \ldots.$$

where

$$G_k^{**} = \frac{\sum_i g_{k/i}^{**} w_i^{**} \Phi_i}{\sum_i w_i^{**} \Phi_i} \qquad (k = 0, 1, 2, \ldots)$$

is the relative proportion of egg cells with k f's in the totality of eggs which contribute to the next generation. If there were no asexual reproduction, the zygotic array immediately after fertilization could be generated by the product of male and female gametic arrays, i.e., by

$$(\sum_j G_j^* f^j)(\sum_k G_k^{**} f^k),$$

because self-sterility of the present material ensures outcrossing, making pollination approximately random. The above product may be rearranged to give

$$\sum_i \phi_i f^i \equiv \phi_0 + \phi_1 f + \phi_2 ff + \ldots + \phi_i f^i + \ldots, \ (i = 0, 1, 2, \ldots)$$

where

$$(3) \qquad \phi_i = \sum_{j+k=i} G_j^* G_k^{**} \qquad (j, k = 0, 1, 2, \ldots)$$

is the relative frequency of zygotes with i f's immediately after fertilization.

However, we cannot neglect asexual reproduction and it is necessary to consider the relative contributions made by sexual and asexual reproduction to the next generation. Let M be the mean number of viable seeds per plant, and let b_i be the probability of a randomly chosen viable seed with i f's reaching maturity. Furthermore, we designate by A_i the mean number of plants which are produced asexually from a plant with i f-chromosomes and which reach maturity in the next generation.

With these definitions, the frequency of mature individuals containing i f-chromosomes in the next generation may be given by

$$\Phi_i' = \frac{Mb_i\phi_i + A_i\Phi_i}{\sum_j (Mb_j\phi_j + \mathrm{A}_j\Phi_j)} \qquad (i = 0, 1, 2, \ldots). \tag{4}$$

Thus in order to calculate the expected frequency distribution at equilibrium, we have to know all the parameters involved in segregation and reproduction, namely, the w's, $g_{j/i}$'s, b's, A's and M. If these are given, a set of equilibrium frequencies denoted by $\hat{\Phi}_0, \hat{\Phi}_1, \hat{\Phi}_2, \ldots$
may be worked out by using relation (4) and applying the trial and error method, starting from a set of arbitrary frequencies and repeating calculations until we get

$$\Phi_i' = \Phi_i \equiv \hat{\Phi}_i, \qquad (i = 0, 1, 2, \ldots)$$

with desired accuracy. The stability of equilibrium will usually become evident in the process of iteration.

The equation (4) may be simplified if we assume that A_i is proportional to b_i, namely, genotypes with higher viability tend to have a higher rate of asexual reproduction. If we accept such an assumption, (4) is reduced to

$$\Phi_i' = \frac{b_i(\phi_i + \theta\Phi_i)}{\sum_j \mathrm{b}_j(\phi_j + \theta\Phi_j)}, \qquad (i = 0, 1, 2, \ldots) \tag{5}$$

where θ is the ratio of the relative contributions by asexual and sexual reproduction to form the next generation such that

$$\frac{A_i}{Mb_i} = \theta \tag{6}$$

for all i, i.e., $i = 0, 1, 2, \ldots$. Then at equilibrium we have

$$b_i = \frac{C\hat{\Phi}_i}{\hat{\phi}_i + \theta\hat{\Phi}_i} \tag{7}$$

where $\hat{\phi}_i$ is the equilibrium value of ϕ_i and C is a positive undetermined constant and may be adjusted so that b_0 is unity.

2. *Application of the mathematical model to the actual data*: We will first try to see whether the observed distribution of the f chromosome in natural populations (Table 1) may be explained satisfactorily from the observed mode of its transmission and its effect on fertility as shown in Tables 2, 3 and 4.

If we assume that different genotypes have the same viability, namely

(8) $$b_0 = b_1 = b_2 = \ldots$$

and that reproduction is almost exclusively sexual, i.e.

(9) $$\theta = 0 \qquad (i = 0, 1, 2, \ldots)$$

then we can calculate the equilibrium distribution using (4) or (5), taking

(10)
$$w_0^* = 0.9525 \quad w_1^* = 0.8767 \quad w_2^* = 0.5326 \quad w_3^* = w_4^* = \ldots = 0$$
$$g_{0/0}^* = 1.00 \quad g_{0/1}^* = 0.50 \quad g_{0/2}^* = 0.07 \quad \ldots$$
$$g_{1/0}^* = 0 \quad g_{1/1}^* = 0.50 \quad g_{1/2}^* = 0.88 \quad \ldots$$
$$g_{2/0}^* = 0 \quad g_{2/1}^* = 0 \quad g_{2/2}^* = 0.05 \quad \ldots$$

with respect to the pollen parent, and

(11)
$$w_0^{**} = 0.6159 \quad w_1^{**} = 0.5229 \quad w_2^{**} = 0.3552 \quad w_3^{**} = w_4^{**} = \ldots = 0$$
$$g_{0/0}^{**} = 1.00 \quad g_{0/1}^{**} = 0.20 \quad g_{0/2}^{**} = 0.05 \quad \ldots$$
$$g_{1/0}^{**} = 0 \quad g_{1/1}^{**} = 0.80 \quad g_{1/2}^{**} = 0.76 \quad \ldots$$
$$g_{2/0}^{**} = 0 \quad g_{2/1}^{**} = 0 \quad g_{2/2}^{**} = 0.18 \quad \ldots$$
$$g_{3/0}^{**} = 0 \quad g_{3/1}^{**} = 0 \quad g_{3/2}^{**} = 0.01 \quad \ldots$$

with respect to the seed parent.

After a considerable number of iterations, the equilibrium distribution (in %) turned out to be as follows:

(12)
$$\hat{\Phi}_0 = 20.2 \quad \hat{\Phi}_1 = 48.4 \quad \hat{\Phi}_2 = 28.9 \quad \hat{\Phi}_3 = 2.4$$
$$\hat{\Phi}_4 = 0.1 \quad \hat{\Phi}_5 = \hat{\Phi}_6 = \ldots = 0.$$

This expected distribution may be compared with the observed distribution in population I, from which materials for the study of segregation and fertility were obtained. In Table 6, classes with the number of f_1 larger than two are

TABLE 6

Observed and expected frequency distributions in population I, assuming equal viabilities of individuals with different numbers of f_1 chromosomes. $\chi^2 = 5.2$, df 3, $0.1 < P < 0.2$

No. of f_1	Frequencies (numbers) Observed	Expected
0	17	11.92
1	30	28.56
2	12	17.05
3 and over	0	1.47
Total	59	59.00

grouped together. Application of the x^2 test gives the x^2 value of 5.2. With three degrees of freedom, $0.1 < P < 0.2$, and therefore the deviation of observed frequencies from the expected is not significant. However, this does not prove that different genotypes have the same viability. On the contrary, Table 6 suggests that individuals having two or more f chromosomes are less viable than those having one f chromosome or none. In fact, if we add all the observed frequencies from four populations and compare the result with the expected, the deviation is now highly significant ($x^2 = 46.4$, df 3, $P < 0.01$). Observed frequencies of individuals having two or more f's are definitely smaller than the corresponding expected frequencies.

However, it might not be desirable to add the observed frequencies from four populations, since the four samples from populations I, II, III and IV are heterogeneous ($x^2 = 19.3$, df 6, $P < 0.01$). The main source of heterogeneity comes from the fact that the two groups of samples I + II and III + IV have different distributions ($x^2 = 17.2$, df 2, $P < 0.01$), though within each group the difference between samples is not significant. If we test the observed frequency distribution of I + II against the expectation, the deviation of the observed from expected is significant ($x^2 = 7.84$, df 3, $P < 0.05$), again showing that those having two or more f's have lower viabilities than those with one or no f.

Therefore, we have to reject the null hypothesis that individuals with different numbers of f chromosomes have the same viability, i.e., the assumption (8) must be abandoned. It may be noted here that though the expected distribution given in (12) is obtained by taking $\theta = 0$, actually the distribution is independent of the value of θ, unless $\theta = \infty$, i.e., the reproduction is entirely asexual.

Having rejected the above hypothesis, next step is evidently to estimate the relative viabilities of individuals with different numbers of f's. For this purpose we assume that the observed populations I, II, III and IV are in equilibrium. The procedure is as follows:

For the sample from population I, we have

$$\hat{\Phi}_0 = 17/59 = 0.2881, \quad \hat{\Phi}_1 = 30/59 = 0.5085$$
$$\hat{\Phi}_2 = 12/59 = 0.2034, \quad \hat{\Phi}_3 = \hat{\Phi}_4 = \ldots = 0.$$

Then using relations (2) and (3), we obtain

$$\hat{\phi}_0 = 0.2769, \quad \hat{\phi}_1 = 0.4908, \quad \hat{\phi}_2 = 0.2177, \quad \hat{\phi}_3 = 0.0139,$$
$$\hat{\phi}_4 = 0.0007, \quad \hat{\phi}_5 = \hat{\phi}_6 = \ldots = 0.$$

The relative viabilities are computed from formula (7) if we know the value of θ, as exemplified in Table 7. Unfortunately no experimental or observational data are available which allow us to determine the value of θ with confidence. Indeed, we have never encountered a report that such a value was determined in any perennial plant species in its natural habitat. However, there are some reasons to believe that, in *L. callosum*, the average value of θ is of the order of a few percent. The senior author is informed by MR. M. SHIMIZU, a lily specialist

in Japan, that most of the lily species, except for the triploid tiger lily, appear to propagate predominantly by seeds. He guessed that the relative contribution made by asexual reproduction might be around five percent in a wild state. It is interesting to find that this figure is substantiated by the following calculation based on our observational data.

From the inspection of Figure 1a, it becomes immediately evident that there is considerable similarity in chromosome constitution between neighboring individuals. By taking, rather arbitrarily, 40 random pairs of nearby individuals in Figure 1a (see Table 8) and calculating the intraclass correlation, we have $r = 0.347$. In order to get a more reliable estimate, the field observation must be specially designed to have a better sample of pairs.

Such a correlation is probably due to one of the following two causes: (a) The neighboring individuals are derived asexually from a same individual through division of bulbs, in which case the similarity is perfect, i.e., the correlation coefficient is unity. (b) The neighboring individuals are derived sexually but are close relatives, such as sibs or more likely half-sibs from the same seed parent.

If we assume, as a first approximation, that the adjacent individuals are either derived asexually from the same bulb or sexually from the same seed parent, the

TABLE 7

Relative viabilities of individuals with various numbers of f_1's. In this table θ represents the ratio between the contributions made by asexual and sexual reproduction to form the next generation

Mode of reproduction No. of f_1	$\theta = 0$	$\theta = 0.1$	$\theta = 1$
0	1.000	1.000	1.000
1	0.996	0.996	0.998
2	0.898	0.907	0.947
3 and over	0.000	0.000	0.000

TABLE 8

Intraclass correlation in the number of f chromosomes between two nearby individuals randomly sampled from population I in Figure 1a. In this table, the symbol (i, j) *denotes the pair of individuals with* i *and* j *f-chromosomes*

Pairs	No. observed	
(0,0)	6	
(0,1)	7	$r = 0.347$
(0,2)	3	$z = 0.362$
(1,1)	13	$\sigma_z = 0.161$
(1,2)	7	
(2,2)	4	
	Total n = 40	

correlation coefficient in the number of the f chromosomes between the adjacent individuals may be given by

$$r = \frac{\sigma_b^2}{\sigma_w^2 + \sigma_b^2} \quad , \tag{13}$$

where σ_b^2 is the variance between sibs (including the case of asexual reproduction which occurs at the proportion of $\theta/(1+\theta)$ of the total), i.e.

$$\sigma_b^2 = \frac{\sum_i M_i^2 \Phi_i w_i^{**}}{\sum_i \Phi_i w_i^{**}} - \overline{M}^2 \quad (\overline{M}\text{: mean of } M_i\text{'s}) \tag{14}$$

and σ_w^2 is the variance within sibs, i.e.

$$\sigma_w^2 = \frac{\sum_i \sigma_i^2 \Phi_i w_i^{**}}{\sum_i \Phi_i w_i^{**}} \quad , \tag{15}$$

in which

$$M_i = \frac{\sum\limits_{j+k=0}^{\infty} (j+k)\ g_{j/i}^{**} G_k^* b_{j+k} + \theta i b_i}{\sum\limits_{j+k=0} g_{j/i}^{**} G_k^* b_{j+k} + \theta b_i} \quad (i = 0, 1, 2, \ldots)$$

and

$$\sigma_i^2 = \frac{\sum\limits_{j+k=0}^{\infty} (j+k)^2\ g_{j/i}^{**} G_k^* b_{j+k} + \theta i^2 b_i}{\sum\limits_{j+k=0} g_{j/i}^{**} G_k^* b_{j+k} + \theta b_i} - M_i^2 \quad (i = 0, 1, 2, \ldots)$$

are the mean and the variance of the number of f chromosomes among sibs reaching maturity and which are derived from individuals having i f-chromosomes.

Though the calculation involved is again laborious, it is possible to estimate θ using the above formulae: Using the values in (10) (11) and observed frequencies of

$$\Phi_0 = 0.2881, \quad \Phi_1 = 0.5085, \quad \Phi_2 = 0.2034, \quad \Phi_3 = \Phi_4 = \ldots = 0,$$

the calculation is repeated until we get $r = 0.347$ from (13), the values of θ, and $b_1, b_2, \ldots$ ($b_0 \equiv 1$) being adjusted at every cycle of iteration. The results obtained were as follows:

$$\theta = 0.07, \quad b_0 = 1, \quad b_1 = 0.996, \quad b_2 = 0.905, \quad b_3 = b_4 \ldots = 0.$$

If we put all four samples (in Table 1) together, estimated values of θ and relative viabilities were approximately as follows:

$$\theta = 0.06, \quad b_0 = 1.000, \quad b_1 = 1.066, \quad b_2 = 0.691, \quad b_3 = b_4 = \ldots = 0.$$

In Table 9, frequency distributions, relative fertilities and viabilities are summarized.

TABLE 9

Summary of frequency distributions, relative fertilities and viabilities. The estimated value of θ is approximately 0.06

No. of f chromosomes	0	1	2	3 or more
Observed frequencies (sum of four samples) Φ	0.333	0.541	0.126	0.000
Expected frequencies at fertilization ϕ (estimated)	0.323	0.489	0.180	0.008
Pollen fertility (observed) w^*	1.000	0.920	0.559	....
Fertility as seed parents (observed) w^{**}	1.000	0.849	0.577	....
Relative viability (estimated) b	1.000	1.066	0.691	0.000

DISCUSSION AND CONCLUSION

Although a considerable number of cases of supernumerary chromosomes have been reported in populations of various species, the present case is rather exceptional for its simplicity of frequency distribution and completeness of experimental and observational data which enable mathematical analysis based on a theoretical model.

The result of the present analysis has shown that individuals with f chromosomes are not only less fertile but also less viable than those with no f chromosomes. This deleterious effect of f chromosomes on viability is especially evident for the case of two or more f chromosomes: With two f's viability is about 70 percent and with more than two f's, it is practically zero. On the other hand for the case of one f chromosome, there is no definite indication of lowered viability. It is possible that even a slight increased viability may exist in plants with a single f, as suggested by the result of analysis on the four samples together. Furthermore, the difference in the details of frequency distribution between samples from different localities such as I + II *vs.* III + IV mentioned earlier, may very well be due to difference in ecologic conditions which influence viabilities of individuals with various numbers of f chromosomes. BOSEMARK (1956) reported

an interesting observation that in *Festuca pratensis*, there was a marked correlation between frequency of accessory chromosomes and the clay content of the soil in Sweden.

Whatever modification is made in the selective pattern by local ecologic conditions, the conclusion is inevitable that in *Lilium callosum* the f chromosome is predominantly deleterious and it may only be kept in the population by preferential segregation in embryo sac mother cells.

Analogous situations occur with the t allele found by DUNN in the mouse (DUNN 1957) and SD factor discovered by HIRAIZUMI in *Drosophila melanogaster* (HIRAIZUMI, SANDLER and CROW 1960). In the former, the t allele (actually consisting of multiple alleles) is a recessive gene located on an autosome and is lethal (or causes complete sterility) when homozygous. Nevertheless, it is found with very high frequencies in wild populations of mice sampled in USA, due to excessive frequency of the t allele among the sperms produced by heterozygous males ($t/+$). If we designate by k (> 0.5) the segregation proportion of t among the sperms produced from $t/+$ males, and if we assume that the heterozygotes, either male or female, have completely normal reproductive ability and that t/t is lethal, then it is possible to show that in equilibrium the proportion of t allele among the pools of male and female gametes are

$$k - \sqrt{k(1-k)} \quad \text{and} \quad \frac{1}{2k}\left\{ k - \sqrt{k(1-k)} \right\}$$

respectively. Thus the proportion of zygotes which fail to develop is

$$\frac{1}{2k}\left\{ k - \sqrt{k(1-k)} \right\}^2$$

or

$$\frac{1}{2}\left\{ 1 - 2\sqrt{k(1-k)} \right\}$$

In other words, this is the proportion of individuals which have to be eliminated to maintain the deleterious allele in the population. Recently the senior author proposed the term distortional load (or load due to meiotic drive) to designate such a load (KIMURA 1960). In the present case, this is given by

$$L_{SD} = \frac{1}{2}\left\{ 1 - 2\sqrt{k(1-k)} \right\}$$

According to DUNN, segregation percentage of t ranges from 89 percent to 99 percent ($k = 0.89$–0.99), so that $\hat{L}_{SD}$ lies between 0.19 and 0.40. This means that in wild populations of mice, even disregarding possible effects of overdominance of t allele, the load due to maintaining t amounts to about 20–40 percent.

A similar concept may be applied to the present case and the distortional load due to f chromosomes may be calculated as follows: From Table 9, the average loss of fertility for plants with one f chromosome is $1 - (0.920 + 0.849)/2$ or 0.115. Similarly for plants with two f's it is 0.432. Thus the component of load due to fertility differences is $0.541 \times 0.115 + 0.126 \times 0.432$ or 0.117, when the population reproduces sexually. The component of load due to viability differences is $0.180 \times 0.309 + 0.08 \times 1$ or 0.064 under sexual reproduction, and 0.126×0.309 or 0.039 under asexual reproduction (see Table 9). In both cases we assume that plants with one f chromosome have the same viability as those with no f's. Combining all these values and taking $\theta = 0.06$, the load becomes $(1 - 0.06)(0.117 + 0.064) + 0.06 \times 0.039$ or 0.1724. Thus in wild populations of *L. callosum*, the distortional load due to f chromosomes amounts to about 17 percent. If apparent increased viability of plants with a single f ($b_1 = 1.066$) is taken into consideration, this value may be increased by three percent.

Though the existence of the increased viability with a single B chromosome is not conclusive in the present case, there may be a considerable possibility for it in a population of organisms in which B chromosomes reach such a high frequency that the majority of individuals carry one or more of them. This is because, under such a circumstance, modifiers which improve viability in combination with B are more advantageous, other things being equal, than those which improve viability without B. If this modification goes on, fitness of individuals with one B will become higher than that with no B's. Through this process, the frequency of B in the population will be gradually enhanced and deleterious effect of B will be diminished. We may say that the B chromosome changes from "parasitic" to "symbiotic."

SUMMARY

1. In *Lilium callosum*, a supernumerary chromosome (B chromosome) denoted by f_1 has a tendency to increase its number due to preferential segregation in embryo-sac mother cells in such a way that in plants with one f_1 chromosome, it moves to the micropylar side in 80 percent of the cases to be included in the egg cells. No such tendency was found in its transmission through the pollen. (KAYANO 1956b, 1957).

2. By crossing experiments involving plants with various numbers of f_1 chromosomes, the mode of transmission and its effect on pollen and seed fertility were investigated. It was found that f_1 chromosomes, when more than one is present, markedly reduced both pollen and seed fertility (Tables 2 and 3).

3. In order to study the frequency distribution of the number of f_1 chromosomes among individuals in natural populations of this lily, samples were collected from four localities in Kyushu and their chromosome constitutions were determined.

The result of this study showed that the frequencies of f_1 are uniformly high: Individuals with one f_1 are most frequent and they together with those having two f_1's compose nearly 70 percent of a population on the average. On the other hand, no individuals were found with more than two f_1's (Table 1, Figure 1 a–d).

4. A mathematical theory was developed to analyse the mechanism of distribution of f_1 chromosomes in the population. The theory contains two new features which do not appear in the ordinary mathematical treatment in population genetics, in that it takes into account of (a) the existence of both sexual and asexual reproduction and (b) the fact that the mode of segregation of the f_1 chromosome does not follow the simple rules characteristic of diploid loci.

5. The analysis has shown that individuals with f_1 chromosomes are not only less fertile but also less viable than those with no f_1 chromosomes. This deleterious effect of f_1 chromosome on viability is especially evident for the case of two or more f_1's. Namely, with two f_1's viability is about 70 percent and with more than two, it is practically zero. For the case of one f_1, there exists no definite indication of lowered viability and it is suspected that there is even a slight increase.

6. The concept of distortional load (or load due to meiotic drive) was applied to the maintenance of f_1 chromosomes in natural populations of *L. callosum* and it was estimated that this load amounts to about 17 percent. A mechanism was suggested by which the deleterious effect of f_1 chromosome may be reduced in the course of evolution.

ACKNOWLEDGMENTS

We would like to express our indebtedness to DR. T. HAGA under whose guidance the experimental and observational work by the junior author had been carried out. Thanks are also due to DR. J. F. CROW who kindly reviewed the draft and made valuable suggestions for improving the manuscript.

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THE FREQUENCIES OF SEED AND SEEDLING ABNORMALITIES IN MAIZE[1]

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Received August 28, 1961

THE fact that cross-fertilizing species carry relatively large numbers of deleterious recessives in a heterozygous condition has been demonstrated by various investigators and for several species. The results obtained have been used to make inferences or to provide a critical interpretation relative to population structure and size, types of gene action, mutation rates, selective advantage, and other attributes which contribute to the description of a population in genetic terms. Relatively little information is available, however, for the subsequent behavior of populations that have been experimentally purged of deleterious recessives. The purpose of the present paper is to provide information about one such case in maize.

Information on the frequency of deleterious recessives is most extensive for Drosophila species. Less information is available for many other species of animals and plants. We shall be concerned here with only the pertinent information available for maize.

JENKINS (1924) reported a range of 0.0 to 35.5 percent for chlorophyll-defective types in first selfed-generation progenies derived from 16 open pollinated varieties. In samples representing six varieties, HAYES and BREWBAKER (1924) reported frequencies of chlorophyll-deficient types ranging from 10.0 to 39.4 percent.

WENTZ and GOODSELL (1929) sampled 19 varieties which had been included in the Iowa State yield test for 1924 and 1925. The observed range for percentage of chlorophyll deficiencies was 3.6 to 42.8. In three varieties, WOODWORTH (1929) observed the percentage of self-pollinated ears segregating for chlorophyll deficiencies to range from 10.0 to 28.9. In a later summary WOODWORTH and MUMM (1931) reported 17.7 percent of self-pollinated ears of the variety Reid segregated for chlorophyll deficiencies and 3.9 percent for defective seeds. This report is of particular interest since a reconstituted Reid was produced by compositing remnant seed of all selfed ears whose progeny were free from detectable segregations. This reconstituted variety was propagated under open-pollination

[1] Contribution from the Iowa Agricultural and Home Economics Experiment Station and the Crops Research Division, Agricultural Research Service, U.S. Department of Agriculture, cooperating. Journal Paper J-4162 of the Iowa Agricultural and Home Economics Experiment Station, Project 1140.

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conditions for a three-year period. At the end of this period the reconstituted variety was sampled and the observed frequencies of chlorophyll deficiencies and defective seeds were found to be 28.6 and 12.2 percent, respectively. These figures represent nearly a twofold increase in chlorophyll deficient types and a threefold increase in defective seeds. No tests for allelism were made in either sample; therefore no estimates of gene frequency were obtained, and increase in frequency of seed and seedling segregations would not have been predicted. The present study was initiated to determine the generality of these results.

MATERIALS AND METHODS

A strain of the variety Reid, maintained by open pollination at the Iowa Agricultural Experiment Station, was used as source material for this study. In a special planting made in 1948 all plants which silked and tasseled at the same time were self-pollinated. Ears from a few plants were lost because of loss of or damage to the shoot bag or because of disease. The 801 self-pollinated ears available for study were assumed to constitute a random sample of the variety.

Each of the self-pollinated ears was shelled individually and a 50 kernel sample grown in a sandbench and examined for seedling segregations. Field plantings were made for all progenies classified as segregating. S_2 seed was obtained to confirm the tentative classification and to provide adequate material for subsequent tests for allelism.

Tests for allelism were made by direct intercrossing of the viable recessive types and classification of the resulting F_1 progeny. Progenies carrying lethals were grown in paired rows involving all possible combinations among similar phenotypes. Within each pair of rows as many intercrosses were made as stand and similarity of time of flowering would permit. With a monogenic character, 4/9 of such matings should exhibit segregation if plants of the two stocks were *AA* and *Aa* in expected frequency and carried members of the same allelic series. If positive indication of allelism was obtained, no further plantings were grown. If no segregation was observed, plantings were repeated until at least ten crossed progenies were available for classification.

After the initial classification for segregation was completed a reconstituted variety was prepared representing equal quantities of seed from the approximately 550 S_1 ears for which no segregations could be detected. Planting was made in an isolated plot and open pollination was permitted. Approximately 400 ears, chosen at random, were used to perpetuate the "reconstituted" variety. The ears were shelled in bulk and passed repeatedly through a sampler to ensure thorough mixing, and an aliquot was taken for planting a 1-acre or larger block the following season. This process was repeated during each of the four years during which the reconstituted variety was grown.

In 1953 approximately 1100 self-pollinations were made in the reconstituted strain. Seeds from the resulting ears were classified for seedling segregations and those classified as segregating were subjected to additional progeny tests for verification of the segregation and to increase seed for subsequent tests for allelism. Tests for allelism were conducted as described for the 1948 series.

EXPERIMENTAL RESULTS

In the sample from the original variety Reid, the percentages of self-pollinated ears segregating for seedling abnormalities was 18.9 (SCHULER 1952). This figure is in good agreement with percentages reported by other investigators. A list of the different phenotypic groups and their estimated gene frequencies is presented in Table 1. Estimated mutation rates are also presented for the nonviable mutant types.

TABLE 1

Estimated gene frequencies and mutation rates for the various mutant alleles isolated from the original Reid

Character		Allele	Frequency of occurrence	Observed gene frequency	Estimated mutation rate	Estimated μ/s equilibrium
Yellow-green						
Group 1	*yg*	a	2	.0013	.....	1.5×10^{-6}
		b	1	.0006	.....	$.38 \times 10^{-6}$
		c	1	.0006	.....	$.38 \times 10^{-6}$
		d	1	.0006	.....	$.38 \times 10^{-6}$
2		e	5	.0031	9.7×10^{-6}	.....
		f	3	.0019	3.5×10^{-6}	.....
		g	1	.0006	$.38 \times 10^{-6}$	.....
		h	1	.0006	$.38 \times 10^{-6}$	.....
		i	1	.0006	$.38 \times 10^{-6}$	.....
		j	1	.0006	$.38 \times 10^{-6}$	.....
3		k	10	.0062	38.9×10^{-6}	.....
		l	1	.0006	$.38 \times 10^{-6}$	.....
		m	1	.0006	$.38 \times 10^{-6}$	.....
4		n	4	.0025	6.2×10^{-6}	.....
		o	2	.0013	1.5×10^{-6}	.....
		p	2	.0013	1.5×10^{-6}	.....
		q	1	.0006	$.38 \times 10^{-6}$	.....
		r	1	.0006	$.38 \times 10^{-6}$	.....
		s	1	.0006	$.38 \times 10^{-6}$	.....
Luteus	*l*	a	7	.0044	19.1×10^{-6}	.....
		b	2	.0013	1.5×10^{-6}	.....
		c	1	.0006	$.38 \times 10^{-6}$	.....
		d	1	.0006	$.38 \times 10^{-6}$	.....
		e	1	.0006	$.38 \times 10^{-6}$	.....
Stripe	*str*	a	3	.0019	.....	3.5×10^{-6}
		b	1	.0006	.....	$.38 \times 10^{-6}$
White	*w*	(a)	21	.0131	171.6×10^{-6}	.....
		(b)	1	.0006	$.38 \times 10^{-6}$	.....
Glossy	*gl*	3	4	.0025	.....	6.2×10^{-6}
		13	1	.0006	.....	$.38 \times 10^{-6}$
		(b)	1	.0006	.....	$.38 \times 10^{-6}$
		(c)	1	.0006	.....	$.38 \times 10^{-6}$
Dwarf	*d*	(a)	6	.0038	.....	14.1×10^{-6}
		(b)	1	.0006	.....	$.38 \times 10^{-6}$
Miscellaneous		.	1	.0006	.....	$.38 \times 10^{-6}$
		.	1	.0006	.....	$.38 \times 10^{-6}$
		.	1	.0006	.....	$.38 \times 10^{-6}$

Two items require some explanation. Twenty-one ears of the 801-ear sample segregated for the virescent phenotype. Through accident this material was lost and no tests for allelism are available. Forty ears exhibited segregation for a yellow-green phenotype. Tests for allelism would have required 780 pairs of progeny rows. This number was greater than could be handled with facilities available. The material therefore was divided into four groups.

In an attempt to minimize the total number of crosses required Group 1 included all phenotypes which offered promise of viability. Allocations to the remaining three groups were at random. Intergroup crosses of established alleles were not completed. Thus gene frequencies for this phenotype may be slightly underestimated. Among the remaining phenotypes the estimated gene frequencies ranged from 0.0006 to .0131.

The estimates of mutation rates for all lethals are based on the assumption that the variety was at equilibrium; i.e. $\Delta\ q = 0$. Consequently $Q^2 = \frac{\mu}{s}$ and the mutation rate μ is equal to Q^2 since for all lethals $s = 1$. The mutation rates calculated in this indirect manner and under the assumption of equilibrium ranged from 0.38×10^{-6} to 171.6×10^{-6}.

In the sample derived from the reconstituted strain the frequency of seed and seedling abnormalities among 1,137 selfed progenies was 8.5 percent. This is approximately half the frequency obtained from the original variety sample. Estimates of gene frequency and mutation rates are presented in Table 2.

The estimated gene frequencies ranged from .0004 to .0101. This range was somewhat less than that observed for the Reid sample. Only ten different mutant phenotypes were observed in the Reid sample while 20 different phenotypes were recorded for the reconstituted Reid. Only six of these were common to the two samples. These except the luteus phenotype exhibit rather similar patterns of gene frequency as is illustrated in Table 3.

Tests for genetic identity among loci for the two samples were not attempted with all phenotypes. Within the glossy group, where the appropriate testcrosses were made, the similarity in frequency estimates does not extend to a complete duplication of loci involved. In the Reid sample gl_3 was found four times, gl_{13} once and two additional unnumbered glossies once each. In the reconstituted Reid sample gl_3 was found once, gl_{13} three times and gl_1 was found nine times. This last glossy was not represented in the original sample.

DISCUSSION

One point of particular interest in this study and the earlier summary report of WOODWORTH and MUMM (1931) is the rapidity with which the reconstituted populations accumulated their mutation load. Several factors could conceivably be involved and these will be considered in the following section.

Sources of possible technical errors will be discussed first. All classification of mutants was either done or verified by the same individual. Thus personal bias in the recognition of aberrant types should have been at a minimum. For this

TABLE 2

Estimated gene frequencies, mutation rates, and equilibrium values for seed and seedling types isolated from the reconstituted Reid

Character	Symbol	Frequency of occurrence	Gene frequency	Estimated mutation rate	μ/s equilibrium
Luteus	*l*	1	.0004	1.6×10^{-7}	.....
White (1)	*w*	10	.0044	1.9×10^{-5}	.....
White (2)	*w*	1	.0004	1.6×10^{-7}	.....
Stripe (1)	*str*	1	.0004	.....	1.6×10^{-7}
Stripe (2)	*str*	1	.0004	.....	1.6×10^{-7}
Virescent	*v*	22	.0101	.....	10.2×10^{-5}
Dwarf (1)	*d*	9	.0040	.....	1.6×10^{-5}
Dwarf (2)	*d*	1	.0004	.....	1.6×10^{-7}
Pale green (1)	*pg*	13	.0057	.....	3.2×10^{-5}
Pale green (2)	*pg*	1	.0004	.....	1.6×10^{-7}
Pale green (3)	*pg*	1	.0004	.....	1.6×10^{-7}
Pale green (4)	*pg*	1	.0004	.....	1.6×10^{-7}
Yellow green	*yg*	11	.0044	.....	1.9×10^{-5}
Glossy	gl_1	9	.0040	.....	1.6×10^{-5}
Glossy	gl_2	1	.0004	.....	1.6×10^{-7}
Glossy	gl_{13}	3	.0013	.....	1.7×10^{-6}
Shrunken	sh_1	17	.0074	.....	5.5×10^{-5}
Onion leaf	..	2	.0009	.....	8.1×10^{-6}
Sugary	su_1	1	.0004	.....	1.6×10^{-7}
Adherent	*ad*	2	.0009	.....	8.1×10^{-6}
Redleaf	..	1	.0004	.....	1.6×10^{-7}
Red stripe	..	1	.0004	.....	1.6×10^{-7}
Accessory blade	*ac*	2	.0009	.....	8.1×10^{-6}
White leaf margin	..	1	.0004	.....	1.6×10^{-7}

TABLE 3

Gene frequency ranges for six phenotypes common to the Reid and the reconstituted Reid samplings

Phenotype	Observed frequency ranges within Reid		Reconstituted Reid	
Stripe	.0006	.0019	.0004	....
Luteus	.0006	.0044	.0004	....
Yellow-green	.0006	.0062	.0044	....
White	.0006	.0131	.0004	.0044
Glossy	.0006	.0025	.0004	.0040
Dwarf	.0006	.0038	.0004	.0040

reason it is believed that the abnormalities detected upon sampling of the "reconstituted" Reid variety represent mutations occurring after the formation of this strain rather than undetected or latent segregations. The number of seed or seedling progeny examined from each self-pollinated ear was sufficiently large to provide odds of greater than 1000:1 that one or more mutant phenotypes should be detected if segregation was normal.

Aberrant segregations were of infrequent occurrence in either sample. The one exception to this generality was the class "germless" seeds. Because the limited progeny tests conducted were somewhat erratic, this class of segregants was ignored in both samples.

Exact records were not kept of the number of parental ears involved in each random mating generation. However, as a close approximation we can assume 550 ears for the composite forming the "reconstituted" variety, 400 ears to propagate the strain in 1950, 1951, and 1952, and 1137 ears representing the sampling in 1953. A total of approximately 6000 gametes was involved assuming two parental gametes per ear.

It is felt that the data obtained were influenced little, if any, by inaccurate classification, by failure to detect segregations, or by excessively small populations during the propagation phases. Consequently any genetic explanation for the observed results must involve one or more of the following: (1) a mutation rate materially greater than 10^{-5}, (2) a much larger number of loci than commonly assumed, or (3) a strong selective advantage for the heterozygote.

Generally speaking, the frequency of an allele giving rise to a deleterious or a normal phenotype is some function of its mutation rate and adaptive value and the breeding system involved. A stable equilibrium is eventually reached for lethals and for sublethals; this equilibrium value is simply the mutation rate μ. The equilibrium values for the two populations are given in Tables 1 and 2. The assumption of equilibrium is undoubtedly more realistic for the Reid variety than for the "reconstituted" Reid, which involved only four generations of free interbreeding. One may calculate the number of generations required to attain the observed frequencies in the "reconstituted" variety using the relationship $n\mu = \log e \frac{p_0}{p_n}$ where n equals the number of generations to reach equilibrium, μ equals mutation rate, and p_0 and p_n are the initial and final frequencies respectively. If we consider the allele, white (1), which was found ten times (Table 2), a very large number of generations are indicated as being required to reach this frequency. Either a high initial mutation rate or other undetermined forces must have been operative to achieve the observed frequency in four generations. A comparable situation was observed for other of the mutant phenotypes.

Tests for allelism of the glossy phenotypes were made within and between the two Reid samples as well as with known genetic stocks. Only two types, glossy 3 and 13, occurred in both samples but with differing frequency. Glossy 3 was present in greatest frequency in the Reid sample. If the difference was of a magnitude to be significant it would suggest that the reconstituted strain had failed to reach equilibrium. However the much greater frequency of glossy 13 in the reconstituted sample does not support such an argument. There remains a possibility that differences in mutation rate as well as in equilibrium may be involved. Either or both of these changes could conceivably have an effect on selective advantage.

If a selective disadvantage involving one or both of the homozygotes were

involved, inbreeding would increase the effectiveness of the selection. Assuming random mating and a minimum population size of 400 ears saved in each generation the expected rate of inbreeding would be approximately one percent per year. In the initial generation involving a composite of S_1 seed, however, assortive mating based on similarity in time of blooming may have led to somewhat greater inbreeding values. In any event it would appear that the inbreeding effects would be important only if accompanied by a selective disadvantage.

It is apparent from Tables 1 and 2 that the estimated mutation rates for the lethal phenotypes and the u/s equilibrium values for the viable types are of essentially the same order of magnitude. Such similarity suggests that if the heterozygote enjoys a selective advantage, such advantage could be rather similar for the two classes. It is assumed that in the reconstituted variety each of the loci in question was represented by the AA genotype until a mutation occurred. Following such a mutation, the frequency of the gene a in the next generation becomes $\frac{q(1-sq)}{1-Sp^2-sq^2}$ where S and s represent the selective disadvantages of the AA and aa individuals, respectively. If the aa individuals are selectively eliminated as would be true for a lethal, the preceding formula becomes $\frac{q}{1-Sp^2}$. Any selective disadvantage of the AA type must result largely from a reduction in number of progeny rather than a differential viability. If a selective disadvantage of 0.5 is assumed and it is further assumed that the initial mutation occurred in one of the gametes produced by the 550 parental plants, then the a allele would increase about eightfold during the four generations involved in this study. If a mild selective disadvantage of 0.1 is assumed for the AA genotype. then the frequency of a would increase about 35 percent in the same period. Thus, while a strong selective disadvantage for AA could result in a rapid increase in the frequency of a, the fact that no such disadvantage has been demonstrated (SCHULER 1954; MANGELSDORF 1928; GARBER and ROWLEY 1927) renders this possible explanation for the observed results somewhat unattractive.

Experiments are currently underway to obtain additional information on the magnitude of any selective disadvantage. The longtime inbred line B14 is being used as the parental stock and induced mutations sought by use of UV and various chemical mutagens.

SUMMARY

Equilibrium values (μ/s) and mutation rates were determined for seed and seedling characters in the maize variety Reid. A reconstituted variety was established from S_1 seed from ears exhibiting no detectable segregation. After four generations of open pollination the reconstituted variety was sampled to provide new estimates for mutation rates and equilibrium values. The frequency of detected segregations was approximately half that of the original population. Mutation rates calculated for the original variety were not sufficiently high to account for the observed rate of accumulation of new mutations. A high initial mutation rate alone or in combination with a strong selective advantage for the

heterozygote could account for the observed results. The data available do not permit a critical distinction between these two alternatives.

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THE INFLUENCE OF GENETIC BACKGROUND ON THE FREQUENCY AND THE DIRECTION OF RADIATION-INDUCED MUTATIONS AFFECTING A QUANTITATIVE CHARACTER

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Received September 5, 1961

MANY independent investigations have been concerned with the influence of a random sample of radiation-induced mutations on quantitative traits in diploid organisms (RUSSELL 1957; BURDICK and MUKAI 1958; WALLACE 1958; FALK 1959; JAMES 1960). The results of these investigations have been inconsistent. Some have revealed a positive shift in the mean of the character under study which has been attributed to a high degree of overdominance of induced negative mutations. In contrast, other investigations have uncovered a negative shift in the mean. These latter results have provided little evidence of overdominance but have indicated the presence of many detrimental mutations with small dominant effects. One explanation for the discrepancies may lie in the fact that the phenotypic effect of induced mutations can be influenced by the genic background with which the mutations must interact. An example has been provided by GUSTAFSSON (1951) who found that certain recessive lethals in barley which were detrimental in the heterozygote under a standard environment, became advantageous under a changed environment, but lost this beneficial effect completely when brought into another genotype.

The present investigation is concerned with the following questions: (1) How important is the genetic background in determining whether induced mutations in a polygenic system will result in a positive or a negative shift in the test character? (2) Is the phenotypic effect of induced mutations discernibly influenced by the degree to which an organism is adapted to its environment?

MATERIAL AND METHODS

The study utilized the character of "growth rate," i.e. rate of cell division, in three different strains of yeast.

The phenotypic effect of mutations was studied by comparing the distribution of individual growth rates of members of a population of irradiated cells with the corresponding distribution for an unirradiated control population. With this technique it was possible to obtain an estimate of mutation effect without identi-

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fying or classifying individual mutants. At the same time, the effects of temporary damage to the cells were excluded from the data.

The diploid strains of *Saccharomyces cerevisiae* which were used in these investigations had to fulfill several conditions. For technical reasons, they had to form circular microcolonies of regular outline on solid medium. For investigational purposes, they had to differ in their genetic origin, in the length of exposure to a standard environment, and in their growth rate. Three lines were chosen: (1) a slow-growing strain, No. 32, (2) a strain of intermediate growth rate, No. 432–1, and (3) a fast-growing strain, No. 4840.

The slow strain, No. 32, was respiratory deficient and formed extremely regular microcolonies. This strain originated in our laboratory as a mutant in a respiratory-sufficient line which had previously been inbred for many generations. It was therefore very homozygous but unadapted to growth as a respiratory-deficient strain.

The intermediate strain, No. 432–1, was derived by crossing haploids obtained from the University of Toronto, and from the University of Southern Illinois (C. C. LINDEGREN's laboratory). This strain had been maintained as a diploid in our laboratory since 1952. A single generation of inbreeding preceded its use in this investigation.

The fast strain, No. 4840, was a cross of two haploid strains which had originated in C. C. LINDEGREN's laboratory, and which had been maintained in our laboratory since 1954. The cross was made immediately before the beginning of the experiment. This diploid was probably highly heterozygous. It sporulated well and was free of recessive lethals. Because of its fast growth rate this line may be regarded as the best adapted of the three to the conditions prevailing throughout the present investigation.

Clonal growth rate was chosen as the character for study because it is affected by many mutations and is an "important" quantitative character. Furthermore, it was shown by JAMES (1959) that the method of estimating growth rates from rate of increase in diameter of microcolonies on solid medium is extremely sensitive. The method permits estimates to be made before natural selection of spontaneous mutation in the rest of the gene complement has obliterated any radiation-induced genetic change.

In preparing stock cultures a single-cell isolation was made from each strain. These were incubated and as soon as the size of the resulting clones allowed, 20 new isolations were made from each. The growth rates of the clones resulting from these latter isolations were determined and those clones showing extreme growth values were discarded. The remaining clones were incubated for three days and then kept refrigerated until used.

Each day one of these clones was incubated in aerated nutrient broth at 33.5°C. As soon as the culture had reached the stationary phase, it was divided into five equal parts, diluted, and each sample divided into two 10 ml subsamples. One of these served as a control. The other was irradiated with 200 ergs/mm^2, using a General Electric germicidal UV-lamp (254mμ). This is a dose which has been shown to induce recessive lethal mutations in approxi-

mately 16 percent of the cells (JAMES and CHAPLIN 1956). The yeast suspension was agitated during irradiation.

Immediately after irradiation 12 cells were isolated, together with 12 cells of the corresponding control sample. Four cells were placed in prearranged locations on a droplet of fortified agar medium on an inverted coverslip (JAMES and SPENCER 1958). Each droplet contained two test cells and two control cells. Each coverslip was inverted over a Van Tieghem-cell which contained a drop of water and was then sealed with vaseline. The slides were incubated at 21.7° ± 0.1°C.

A detailed description of the technique for measuring the microcolonies and for calculating the values in which growth rate is expressed, is given by JAMES and SPENCER (1958) and JAMES (1959). We used the so-called two-point value, in which the growth rate of a clone is represented by the average increase per hour of the logarithm of the colony diameter: $\frac{\log^2 - \log^1}{\Delta t}$. All physiological effects, such as division delay and stimulation (BURNS 1958; JAMES and MÜLLER 1961), which might have been caused by the irradiation, were excluded from the data by this method. The two measurements on which the growth value was based were made during the logarithmic phase of growth. At this time the growth curve of the colony was linear on a semilogarithmic scale. The time interval between the two readings was approximately the same for all clones of one strain. This interval differed between strains because of their different rates of growth.

No attempt was made to differentiate between those cells that divided only a few times and then stopped and those that did not reach the lower limit of the linear part of the growth curves at the time of the first measurement. Both appear as "near-lethals" in the graphs. In the slow strain, No. 32, many test and control cells showed such a considerable lag before the first division and such a slow growth afterwards, that the colony size did not permit an exact measurement. Because this lag was indistinguishable from irradiation-induced lag and slow growth, the number of near-lethals in this strain was not determined. All doubtful cases were excluded from the calculations and graphs. There were always some cells which did not divide after planting because they had been killed either by irradiation or by micromanipulation. For the statistical analyses only paired data were used.

Data from approximately 500 pairs of irradiated and control cells were obtained for each strain. The distributions of growth rates were each compiled from 12 to 16 daily lots of data. Small differences in such factors as temperature, humidity, subculture, and medium, can affect the means of the lots significantly. A correction was applied to prevent such differences from increasing the variances and obscuring differences between the control and irradiated samples. Here the overall mean growth value of the controls was taken as a standard. The differences between this and the daily control means provided a value for correcting all individual control and irradiated values. Overall variances were calculated by adding the "daily" sums of squares and dividing by the sum of the degrees of freedom. All other statistical methods were standard.

RESULTS

The results are presented graphically in Figures 1, 2 and 3, and statistical analyses are summarized in Table 1. An inspection of the figures is sufficient to

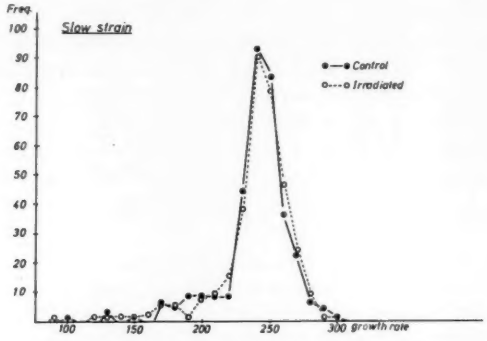

FIGURE 1.—Distribution of growth rates within an irradiated and a control population of the slow strain, No. 32.

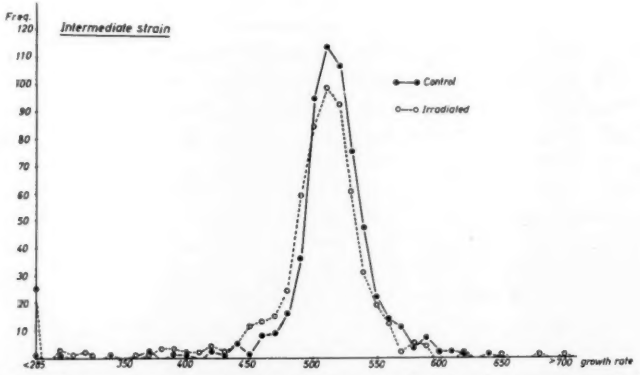

FIGURE 2.—Distribution of growth rates within an irradiated and a control population of the intermediate strain, No. 432–1.

TABLE 1

Statistical analyses of the effect of irradiation on subsequent growth of cells of a slow, an intermediate, and a fast-growing strain of yeast

		Slow strain no. 32				Intermediate strain no. 432–1				Fast strain no. 4840			
		Cont.	Irr.	n	P	Cont.	Irr.	n	P	Cont.	Irr.	n	P
Mean growth rate	(a)*	239.8	240.5	336	>0.8	515.4	504.4	561	<0.001	568.1	571.4	540	>0.1
	(b)	....	....	...	..	514.6	493.5	584	<0.001	566.7	564.8	555	>0.5
Modal value		240	240	336	..	510	510	584	....	570	570	555	....
Variance	(a)	690.4	636.6	336	>0.20	937.8	1940.3	561	<0.001	1483.2	2388.3	540	<0.001

* a=without near-lethals, b=with near-lethals.

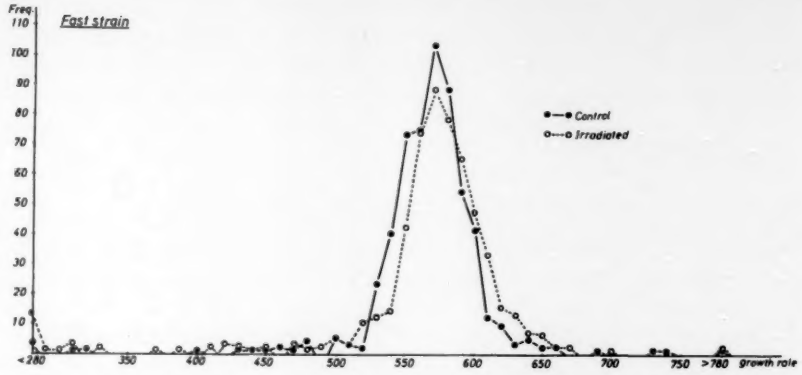

FIGURE 3.—Distribution of growth rates within an irradiated and a control population of the fast strain, No. 4840.

show that the investigation was successful in demonstrating irradiation-induced shifts in a polygenic trait. The direction of these shifts is, however, somewhat surprising. Taken at face value, the data imply that mutations have been: (a) completely ineffective in the slowest strain, (b) negative in the intermediate strain and (c) mainly positive in the fastest growing strain.

The data from each strain will be considered separately in more detail.

Slow strain: It is very doubtful if any induced mutations were able to improve growth rate in the slow strain, No. 32 (Figure 1). Certainly the number of such mutations must be very small. On the other hand, there were also few or no variants showing a small detrimental effect; the mean, mode and variances of control and irradiated populations are practically the same. Nothing can be said about the occurrence of near-lethals since no attempt was made to detect them, but it can be suspected that some were induced.

Intermediate strain: In contrast, there can be no doubt that irradiation induced a relatively high number of mutations with detrimental effects in the intermediate strain (Figure 2). The modal values of both samples are the same, but the ratio of colonies to the right and to the left of the mode is significantly different: $\chi^2 = 10.99$; $P < 0.001$ (without near-lethals). The same fact is reflected in the significantly lower mean of the irradiated population (Table 1). Even if the pairs with a near-lethal partner are not included in the calculation, the mean remains lower.

It seems that most of the increase in the variance in the intermediate strain is due to negative mutations. Nevertheless, to find out if some positive mutations occurred as well, the variances of those sections of the two distributions which are to the right and to the left of the mode were compared. In effect, the histograms were dissected at the mode and the halves rotated on this axis to produce four completely symmetrical curves. A comparison of these variances (Table 2) showed that most of the difference between the total variances is attributable to

TABLE 2

Variance analyses of the effect of irradiation on the distribution of growth values of the intermediate strain, no. 432–1

	Var. control	Var. irrad.	F	P
Total	40.59	61.71	1.52	<0.01
Minus side of mode	83.78	150.92	1.80	<0.01
Plus side of mode	61.78	69.56	1.13	>0.05

the minus side of the distribution. There is only a small and insignificant increase in the plus variance of the irradiated sample, a fact which shows that there are few, if any, positive variants.

Fast strain: The results for the last and fastest growing strain, No. 4840, are different from those for either of the other two strains (Figure 3). Here the most striking characteristic of the irradiation effect is the shift of the exposed population to the positive side. Even the modal value of the distribution is shifted to the next higher class when the number of classes is doubled. This shift is in contrast to the absence of any such effect in the first two strains, where the test modes remain in the same class as the control modes, regardless of any reduction in the size of the class interval. The variance of the values from the irradiated population is significantly larger. Irradiation did not affect the mean of this strain (Table 1), a fact which, in view of the increased variance, indicates that negative variants, as well as positive, were induced.

The increase in the modal value for the fast strain upon irradiation is so large as to be disconcerting; it implies that a positive mutation occurred in nearly every one of the irradiated cells. Even if it is assumed that the positive shift of the modal value is only a matter of chance, then still an improbably high number of positive variants remain. As a consequence, the data were examined carefully to see whether there could be some explanation for this phenomenon other than mutation.

A systematical error in the experimental method is suggested by the regular appearance of the curve. Such an error might result from a convexity of the growth curves when log diameter is plotted against time. Due to the induced delay of the first cell-divisions, the irradiated clones did not usually reach the same diameter as the controls in the time interval between planting and measurement. As a result, the average diameter at the second measurement was higher in the control than in the irradiated sample. This fact would not have any influence on the estimated growth rates if all measurements were taken on the linear parts of the growth curves. However, if the larger colonies lie on a declining curve, then their estimated growth values will be too low. It was shown by JAMES (1959), that the growth curves of colonies are nearly linear on a semilogarithmic scale between diameters of 0.1 and 1.0 mm. With the exception of a few colonies larger than 1.0 mm all our measurements were made between the above mentioned limits. Nevertheless, it seemed possible that a slight convexity would lead to a positive shift. The existence of such a bias would be of minor

importance in the cases of the slow and intermediate strains, but might be of major importance in interpreting the results of irradiating the fast strain.

Five independent tests were undertaken in an effort to determine the extent of the separate influences of mutation and of curvature on the growth values of the fast growing strain.

(1) In the first test direct evidence of curvature was sought by comparing two growth values obtained from the same clone at different times. Each clone was measured three times. The interval between the last two measurements was two hours, a time in which a decline of the curve should show up clearly. The growth values calculated from this interval should be lower on the average, than those from the first interval if the data are in fact biased by nonlinearity of the growth curves. This was certainly not the case; in 19 of 32 control clones, and in 21 of 32 test clones the growth values calculated from the second interval were actually greater than those from the first interval.

(2) A second test for nonlinearity of the growth curves was based on the expectation that there would be a negative correlation between the size of the colony at the time of the final measurement and its growth value if the curve is nonlinear. The regression coefficients from three randomly chosen lots revealed no such relation:

$$\text{Lot 1: } b = -47.67 \pm 40.31$$
$$\text{Lot 2: } b = +91.66 \pm 54.61$$
$$\text{Lot 3: } b = +83.07 \pm 32.00$$

Only the third coefficient is significant, and this is a positive regression. The existence of such a positive regression is not surprising, because the fast growing colonies naturally tend to be the larger ones.

(3) In the third test an attempt was made to remove any bias introduced by nonlinearity and then to see if a positive shift remained. All data were divided into several classes according to colony size at the final measurement (log 2). Growth rate comparisons were then made within each class. Figure 4B shows the mean growth rates in each of these classes. There is a distinct drop in the mean growth rate of the classes containing larger colonies. This is without doubt due to flattening of the growth curves. However, the number of clones in these classes is too small to play a decisive role in the distribution, as can be seen from Figure 4A, which shows the frequency of clones in each class. All values from classes greater than 2.0 were excluded from further calculations. In the other classes the control means usually lie below the means of the irradiated sample. A χ^2 test over the 13 classes in which both groups are represented by more than five clones, implied a significant difference ($P < 0.02$). It is evident that irradiation has affected the growth values regardless of any bias attributable to nonlinearity of the curves.

(4) The fourth test succeeded in showing that nonlinearity in the growth curves also contributes to the positive shift in the irradiated sample (despite indications to the contrary in the two initial tests). This was accomplished through the detection of nonlinearity within control curves. From all control pairs (1

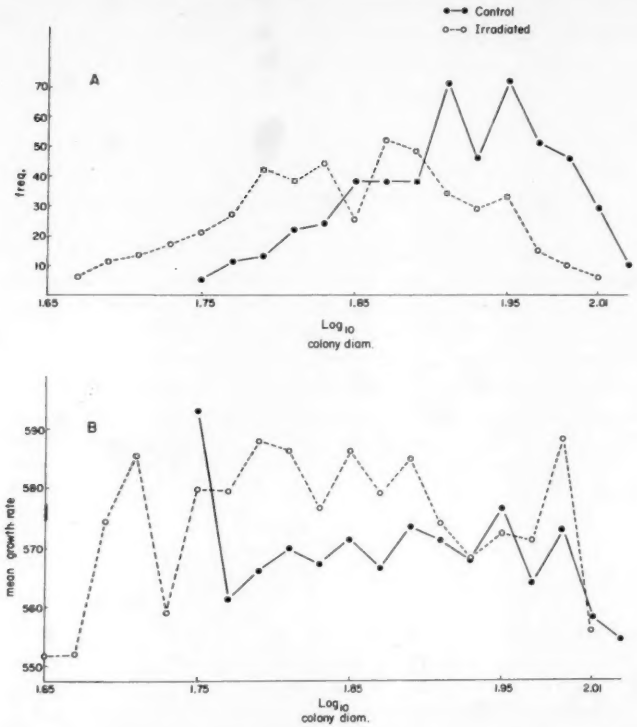

FIGURE 4.—(A) Frequencies of irradiated and control clones of No. 4840, when classed according to their size at the final measurement. (B) Mean growth values of clones in each class.

pair in each Van Tieghem-cell) the partners with the larger diameter were classed as one group. Their growth values were subtracted from the values of their partners which were smaller. In the absence of a bias, the resulting distribution of differences should be a normal one around the modal value 0. But Figure 5A shows that it is even more skewed than the distribution of the values for irradiated minus control colony (Figure 5B). Distribution 5C, which is essentially normal, is obtained if one control value is subtracted randomly from the other without regard to colony size. It is clear that a bias exists. Whereas the initial tests showed that the absolute size of a colony has no influence, within certain limits, on its growth value, this more refined test makes it obvious that the relative size is important; where one clone is larger than its neighbor its growth value tends to be lower.

(5) The demonstration of a bias due to nonlinearity of growth curves does not, of course, negate the evidence (test 3) that a positive shift exists independently of that bias. In fact, it was possible to corroborate the existence of the "muta-

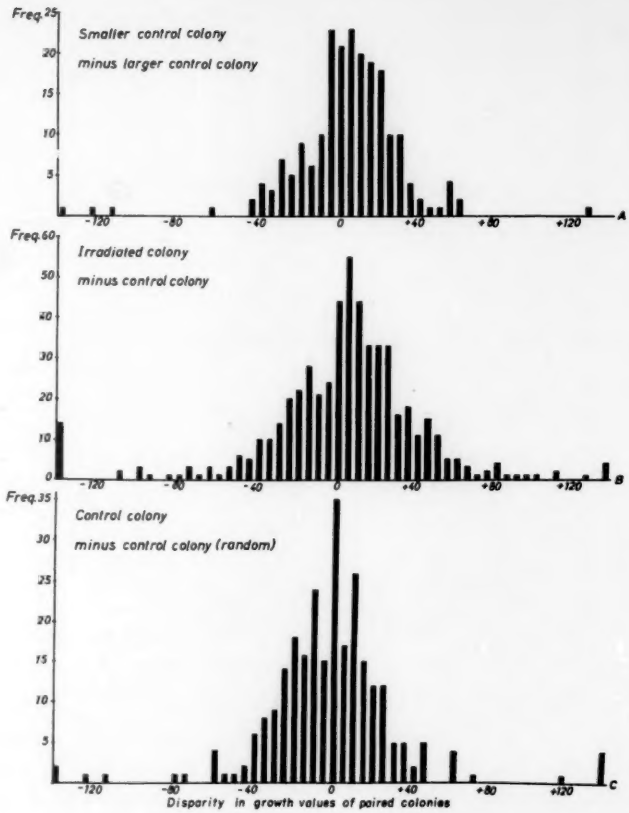

FIGURE 5.—Distributions of differences between paired growth values.

tional" shift with the same general technique used to demonstrate the bias. The reasoning was as follows: The distribution of a sample of control growth value differences where half the subtracted values are from larger colonies and half are from smaller colonies is essentially normal (Figure 5C). If the irradiation-induced positive shift is caused only by the bias, then the distribution of the differences (irradiated minus control) should be skewed to the minus side when it is artificially so composed that the irradiated clone is always larger than the control. Similarly, the distribution of these values should be skewed to the plus side when each irradiated clone is smaller than its control. A distribution artificially so composed that half the irradiated clones are larger and half are smaller should, like that of Figure 5C, be essentially normal.

Figure 6 shows the distribution actually obtained. Where the irradiated clone

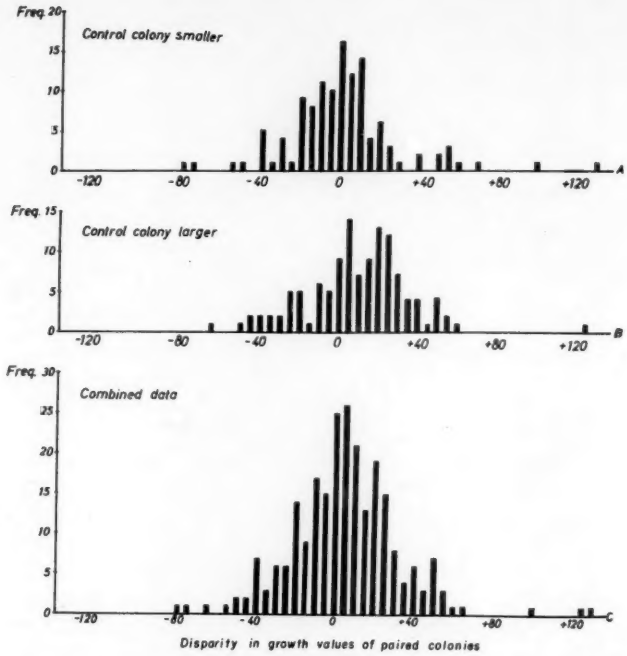

FIGURE 6.—Distribution of differences in growth values between irradiated and control colonies.

is the larger (6A), the distribution is not skewed but is almost symmetrical, 61 minus:59 plus, about the modal value of zero. Figure 6B represents a sample of the same size as 6A, selected randomly out of the large group of pairs where the controls were larger than their irradiated partners. It shows that the positive shift, 36.5 minus:83.5 plus, is much stronger than the corresponding control shift (Figure 5A) with 73 minus:136 plus. The sum of both distributions (6A + 6B = 6C) is not normal, but displays instead a significant positive shift, 97.5 minus:142.5 plus. A positive shift of the distribution of test values, independent of the bias discussed above is implied.

It was possible to counter an objection that the random sample in which the control is larger (6B) is not equivalent to that in which the control is smaller (6A). Such a lack of equivalence might stem from the fact that the colony responsible for a discrepancy in size can be either test or control. For example, a sample selected because of the large size of its control colonies might give biased information on relative growth rates within the population. The absence of such a bias was demonstrated by comparing the ratios of growth values (minus:plus) of the random sample, 6B, with those of two groups, each of 120 pairs, selected

from the sample with the larger controls. One of these two groups contained the largest controls. The other contained the smallest irradiated clones. They gave proportions of 28.5 minus:91.5 plus and 34.5 minus:85.5 plus, respectively. Both were even more positive than the random sample, but neither was significantly different from it ($P > 0.2$ and > 0.7 respectively). It is evident that the samples are equivalent and that a radiation-induced positive shift exists independently of a methodological bias associated with nonlinearity of the growth curves. In the absence of any other known cause, this must be attributed to positive mutation.

DISCUSSION

Any one of three different categories of results might be expected from this investigation of the phenotypic effects of random samples of radiation-induced mutation in different genetic backgrounds. First, all three strains might have reacted similarly, whatever the effect. This would imply a low degree of epistasis in radiation-induced mutations affecting quantitative characters. Second, the strains might have reacted differently from each other, but in accordance with some simple basic pattern. Such a pattern might be an increased tendency toward a positive shift as the degree of "fitness" is reduced. Certainly it is reasonable to suppose that a random mutation has a better chance of being beneficial if it occurs in a poor strain. Third, the reaction of the strains might have been so complexly individualistic as to appear capricious.

The results of this investigation point unequivocally to this last possibility; they emphasize the overriding importance of particular, and often unrecognized, aspects of the individual genetic background in determining the phenotypic effect of mutant genes. The slowest and least adapted strain was not obviously changed despite its inferior genotype. In contrast, the intermediate strain produced many slow-growing variants, and the fastest and "best adapted" strain produced positive variants in addition to negative variants.

With hindsight it is relatively easy to provide a suitable explanation for the behavior of each of the strains. An explanation of the lack of any effect in the slow strain may lie in the fact that cytoplasmic deficiency acts as a block in the positive direction and as a buffer in the negative one. The decrease in the rate of growth as dictated by the deficient plasma is much larger than the effect of small detrimental genic mutations in an otherwise well functioning genome would be. In all probability the result would have been the same if, instead of this cytoplasmic defect, the strain had possessed one or more detrimental genes which controlled some basic biochemical reactions. It seems likely that the reaction of this strain to irradiation may be duplicated in all those populations where a strong epistatic gene prevents any phenotypic expression of radiation-induced changes in a polygenic system.

The response of the intermediate strain, No. 432-1 to irradiation is of the kind to be expected from a well-adapted population; the probability that any induced mutation will disturb the balance is very high.

The reaction of No. 4840, in producing a significant number of positive variants is more surprising. It can only be assumed that this strain, which was newly

diploid, was still far from having attained optimal adaption; despite its rapid rate of growth, many possibilities for further improvement remained.

There must be factors in addition to gene interaction which affect the genetic response of a strain to irradiation. These include mitotic crossing over and rate of gene mutation. Such factors must share responsibility for the vagaries exhibited by various genetic constitutions. There can be no doubt that an accurate prediction of the phenotypic effect of a random sample of radiation-induced mutations on a quantitative character is impossible in the absence of explicit information about the genetic background against which these mutations are placed.

SUMMARY

The influence of genetic background on the phenotypic effects of a random sample of radiation-induced mutations affecting a quantitative character was studied. The character was rate of cell division in *Saccharomyces cerevisiae*.

Three diploid strains of yeast which differed in rate of cell division and in degree of heterozygosity were utilized. The distributions of growth rates of clones produced by single-cell isolates of the irradiated populations were compared with those for unirradiated control samples.

No two of the strains reacted alike. Mutations were completely ineffective in the most homozygous and least adapted strain, mainly negative in the intermediate strain, and mainly positive in the most heterozygous and fastest growing strain.

It was concluded that vagaries in the influence of different genetic constitutions on the expressions of the mutant genes are stronger than was originally supposed. The importance of epistasis in quantitative inheritance is emphasized.

ACKNOWLEDGMENTS

The senior author wishes to express her thanks to the National Research Council of Canada, which made these studies possible by the grant of a postdoctorate Fellowship, to Atomic Energy of Canada Ltd. for providing research facilities and to DR. H. B. NEWCOMBE for his interest and many helpful suggestions. Finally our thanks are due to MRS. C. TOWNSEND and MR. A. MACDONALD for their technical help.

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EXPERIMENTAL STUDIES ON RELATIVE VIABILITY IN DROSOPHILA MELANOGASTER[1]

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Received September 5, 1961

IN large laboratory populations, changes of gene frequency are generally attributable to the systematic effects of selection. This is especially so in populations maintained at around a constant size of, say, 10,000 adult flies. The total amount of selective elimination in such populations may be a function of any one of a large number of variables, or of any of their theoretically possible combinations, acting at any or several points in the life cycle. To facilitate analysis such a complex may logically be reduced to a small number of categories, such as inviability, infecundity, and selective mating. These may then be said to constitute the components of selection (i.e., of elimination), and their complements to constitute the components of adaptive value, or fitness, provided that the categories are considered in an exhaustive sense.

The present study is an attempt to isolate and measure the inviability component of selection, or speaking complementarily, the viability component of fitness. An attempt is also made to analyze, in a general way, the factors which in turn are responsible for inviability. This course of work was suggested by the intense larval crowding observed in laboratory populations of the alleles wild type and ebony^{11} of *Drosophila melanogaster*, and by the seemingly related decrease in the frequency of the ebony^{11} phenotype. Corresponding phenomena, leading to an ostensibly stable equilibrium, have been reported for the mutants ebony (*e*) (L'HÉRITIER and TESSIER 1937; KALMUS 1945; TESSIER 1947; RASMUSSEN 1958) and ebony-sooty (e^s) (CARSON 1958).

MATERIALS AND METHODS

Wild-type and mutant strains of *Drosophila melanogaster* were used for these experiments, the mutant being the third chromosome recessive ebony^{11} (e^{11}) (name and symbol hereinafter simplified by omission of the superscript). This mutant has good viability and is readily separable from wild-type, properties which, among others, resulted in its being listed as of rank 1 by BRIDGES and BREHME (1944). Both strains were maintained by mass transfer in half-pint culture bottles and were used without any attempt to make them isogenic or to increase their homozygosity. Since a failure in temperature control resulted in

[1] Work aided by U.S. Public Health Service Grant RG-4174 and by funds from the State of Washington Initiative Measure No. 171 for the Support of Biological and Medical Research.

the loss of all flies with which the experiments were begun, new stocks were obtained and certain experiments repeated to provide a comparison between old and new strains (Table 1). The original wild-type strain was caught locally. The

TABLE 1

Comparison of old and new strains using frequencies of ebony from the cross +/e × +/e, under different degrees of crowding

Cross	Strain	Number counted		χ^2		Percent	Differences
		+/−	e/e	(3-1)	(h)	e/e	
1 ×(1)	old	2282	760	<0.1	1.3	24.98	1.20
1 ×(1)	new	3549	1107	3.7		23.78	
1 × 1	old	3126	1034	<0.1	1.1	24.86	1.06
1 × 1	new	2594	810	2.6		23.80	
5 × 5	old	8799	2823	3.1	0.2	24.29	−0.19
5 × 5	new	52832	17126	10.1**		24.48	
100 ×(100)	old	14910	3984	154.4***	3.5	21.09	1.13
100 ×(100)	new	5707	1423	96.7***		19.96	
100 × 100	old	17370	4245	331.3***	31.3***	19.64	2.29
100 × 100	new	19239	4039	726.3***		17.35	
200 × 200	old	8330	1513	486.7***	2.6	15.37	1.27
200 × 200	new	3795	623	279.9***		14.10	

Analysis of variance of the frequencies of ebony

Source	DF	SS	MS	F
Total	11	1645708	.....	....
Parental densities	5	1592040	318408	102.1***
Strains	1	38081	38081	12.2*
Residual	5	15587	3117	....

The average of differences in the right-hand column is 6.76%/6=1.13%. h=heterogeneity. To convert mean squares to decimal equivalents multiply each MS by 10^{-8}.

*, **, and *** indicate statistical significance at the 0.05, 0.01, and 0.001 probability levels, respectively, in all tables in this paper.

other strains, not caught locally, are of uncertain origin; of these, the "new" strains are still available.

Standard corn meal-agar-molasses medium with an addition of dry brewer's yeast (Anheuser-Busch, strain B) and sodium propionate (Dupont, Mycoban), the latter to inhibit mold, was used in all experiments. Before the flies were placed in a culture bottle a suspension of 5 ml of Fleischman's dry yeast in 50 ml of water was added to each culture, the amount of suspension depending upon the size of the bottle and the nature of the experiment. To prevent sticking of flies in the medium a small amount of powdered agar-agar was sprinkled over the surface

whenever necessary. Paper strips, uniform in size for a given size of culture bottle, were placed in all bottles to provide extra pupation surface. All of the earlier work was done at 25 ± 1.5°C, the latter at 25 ± 1°C.

RESULTS

The density series: It was desired to test the possible effect of crowding upon relative viability during preadult life under conditions comparable to those obtaining in a population cage. The simplest way to make this test, it seemed, was to repeat the cross $+/e \times +/e$ under a wide range of densities and to determine the effect upon the frequencies of the resulting progeny, which, if there were no relative inviability, would be expected to occur as $+/+$, $+/e$ and e/e in 1:2:1 ratio. Thus the three genotypes which coexist in a population model would also be present among the progeny of a cross used as a viability test, without being influenced by any possible variations of fecundity or mating system such as might be introduced if the parents were not all $+/e$. Corresponding tests on the viability of various mutants of *D. melanogaster* have been used by LEWIS (1954), employing a density series; and by STERN, CARSON, KINST, NOVITSKI and UPHOFF (1952), KERR and WRIGHT (1954), BURI (1956) and SPOFFORD (1956), not employing a density series since the matter of density was not relevant to the purpose of their experiments.

Following a four-day fertilization period the $+/e$ flies were transferred to pint-bottle test cultures. Each bottle contained 80 ml of medium and a standard quantity of live yeast suspension (1 ml before introduction of the parental flies, 2 ml at the time of their removal seven days later). In some experiments only females, and in others both females and males, were placed in the test cultures; the former are indicated with the males in parentheses (Tables 1, 2 and 3).

TABLE 2

Frequency of heterozygotes among the dominant progeny of the cross +/e × +/e, under different degrees of crowding

Cross	Number tested			Percent +/e	χ^2	
	+/+	+/e	Total		(1:2)	(h)
1 ×(1)	142	302	444	68.02	0.4	
1 × 1	63	134	197	68.02	0.2	
5 × 5	351	750	1101	68.12	1.1	
5 × 5	127	272	399	68.17	0.4	
5 × 5	65	162	227	71.37	2.3	
5 × 5	88	231	319	72.41	4.7*	
5 × 5	114	247	361	68.42	0.5	7.3
100 ×(100)	74	173	247	70.04	1.3	
100 × 100	84	190	274	69.34	0.9	
200 × 200	53	98	151	64.90	0.2	
400 × 400	64	131	195	67.18	<0.1	
800 × 800	80	171	251	68.13	0.2	
1600 × 1600	42	66	108	61.11	1.5	
Total	1347	2927	4274	68.48	6.3*	

h = heterogeneity.

TABLE 3

Numbers, frequencies and viabilities of progeny from the cross +/e × +/e, under different degrees of crowding

Cross	Number of cultures	Numbers counted			χ^2 (3:1)	Frequencies (percent)			Viabilities (percent)		$\hat{V}$
		+/− (D)	e/e (R)	Total (T)		+/+ (α)	+/e (β)	e/e (γ)	+/+ ($2\alpha/\beta$)	e/e ($2\gamma/\beta$)	e/e (V/V_1)
1 ×(1)	20	2282	760	3042	<0.1	24.00	52.15	23.85	92.04	91.48	100.00
5 ×(5)	20	6069	1955	8024	1.7	24.19	52.57	23.23	92.04	88.39	96.62
10 ×(10)	20	9624	3113	12737	2.1	24.17	52.52	23.31	92.04	88.77	97.04
25 ×(25)	20	13940	4531	18471	2.2	24.14	52.46	23.40	92.04	89.21	97.52
50 ×(50)	20	15289	4519	19808	50.5***	24.68	53.63	21.68	92.04	80.86	88.39
100 ×(100)	20	14910	3984	18894	154.4***	25.23	54.82	19.96	92.04	72.81	79.59
150 ×(150)	20	11750	2355	14105	518.7***	26.61	57.82	15.57	92.04	53.84	58.85
200 ×(200)	20	6810	1241	8051	394.6***	27.01	58.70	14.28	92.04	48.67	53.20
1 × 1	20	3126	1034	4160	<0.1	24.04	52.24	23.73	92.04	90.84	99.30
5 × 5	20	8799	2823	11622	3.1	24.22	52.62	23.16	92.04	88.02	96.22
10 × 10	20	13474	4239	17713	10.8**	24.33	52.87	22.80	92.04	86.26	94.29
25 × 25	20	17207	5188	22395	40.2***	24.57	53.39	22.04	92.04	82.54	90.23
50 × 50	20	16591	4901	21492	55.3***	24.69	53.64	21.67	92.04	80.81	88.34
100 × 100	20	17370	4245	21615	331.3***	25.68	55.81	18.51	92.04	66.33	72.51
150 × 150	20	12669	2279	14948	758.5***	27.07	58.82	14.12	92.04	48.00	52.47
200 × 200	20	8330	1513	9843	486.7***	27.03	58.73	14.24	92.04	48.50	53.02
400 × 400	10	2301	369	2670	178.0***	27.16	59.02	13.82	92.04	46.83	51.19
800 × 800	10	2831	339	3170	346.0***	28.15	61.16	10.69	92.04	34.97	38.23
1600 × 1600	5	1777	153	1930	300.0***	29.02	63.05	7.93	92.04	25.14	27.48

Each set of 20 cultures represents data from old stock; calculation of genotype frequencies for these sets includes adjustments for (1) old and new strains and for (2) heterosis, as follows: $\alpha=0.3152(D/T + 0.0113)$, $\beta=0.6848(D/T + 0.0113)$, $\gamma=R/T - 0.0113$. Sets of less than 20 cultures represent data from new stock, for which calculation of genotype frequencies includes adjustment for heterosis only.

Progeny were counted for an eight day period following first emergence and provide the data of columns 3, 4 and 5 of Table 3. Some of the data of Table 3 have been reported previously (MOREE 1952); but since the density series has been considerably extended, and some parts of it repeated, and since the present analysis is made on a genotypic rather than a phenotypic basis, the entire set of data is given.

To make the estimates of frequencies and viabilities given in Table 3 it was first necessary to have information (1) permitting a comparison of old and new strains with respect to the frequency of ebony progeny and (2) providing a measure of the frequency of $+/e$ among the wild-type progeny, both under conditions of variable parental crowding.

Results of tests comparing old and new strains are given in Table 1. Differences between these, with respect to the frequency of ebony offspring, turned out to be less than was expected, with the average frequency of e/e for the new strains being 1.13 percent less than that for the old. Although the analysis of variance shows this slight difference to be significant, the significance may be doubtful in view of the heterogeneity introduced by the 100×100 crosses of the new strains. On the other hand, the new strains produce a lower frequency of e/e in all but the 5×5 crosses, and here the difference is very small. Therefore an adjustment for strain differences is included in the calculations of genotype frequency given in Table 3.

Progeny tests were used to determine the frequency of F_2 $+/e$ under different degrees of crowding. Virgin wild-type females and males, collected from cultures corresponding in degree of parental crowding to those of the density series, were crossed with e/e males and virgin e/e females, respectively. Single-pair matings were made in shell vials and these classified according to whether the tested flies were $+/+$ or $+/e$. The results, given in Table 2, are from the new strains; unfortunately no corresponding tests were made on the old strains. With no heterosis and no differential effect from crowding the expected frequency of $+/e$ would of course be 66.67 percent. In two cases experimental values are below this level and in 11 they are above it, but only in one 5×5 cross is the deviation significant. It is interesting that most of the frequencies of $+/e$ in the series are not significantly altered by crowding. The pooled data give an experimental frequency of 68.48 percent and thus, in a descriptive sense, indicate a small but significant amount of heterosis to be *expressed* in the carriers of the $+/e$ genotype. Such heterotic effects as might have been produced by genes at other loci in the vicinity of e or elsewhere did not cause a great alteration of the frequency of $+/e$ relative to the frequencies of $+/+$ and e/e. On the basis of these results an adjustment for the slight superiority of the heterozygotes is included with that for strain difference in calculating the genotype frequencies given in Table 3.

Examination of the observed numbers, genotype frequencies, and total viabilities in Table 3, in relation to parental crowding, indicates that while crowding must lower the absolute viabilities of all classes of progeny, it lowers that of e/e relatively more than it does that of $+/+$ or $+/e$; and $+/+$ and $+/e$, in consequence of results discussed in the preceding paragraph, are not differentially

affected by crowding. Interclass correlations between degree of parental crowding and frequency of ebony offspring are $r = -0.987$ for the ♀×(♂) crosses and $r = -0.836$ for the ♀×♂ crosses. As regards observed numbers, the optimal condition for mutant survival occurs at the 25-female level of parental crowding; but as regards frequency and viability, the optimal condition occurs when parental crowding is at a minimum. If, at this level of crowding, mutant viability is calculated on a phenotypic rather than on a genotypic basis, and without adjustment for strain difference and heterosis, it is 99.9 percent, practically equal to that of wild type (MOREE 1952; MOREE, NEWHOUSE and KING 1957).

Table 4 gives the results of several analyses of variance for the data of Table 3.

TABLE 4

Variation in the density series. Analysis of variance of the frequencies of ebony from ♀×(♂) crosses (A) and from ♀×♂ crosses (B), the latter calculated for unequal subgroups. Analysis of variance of the pooled frequencies of ebony comparing ♀×(♂) and ♀×♂ crosses; unadjusted (C) and adjusted for strain difference (D)

	Source	DF	SS	MS	F
	Total	159	5651717	.....	
A	Densities	7	3236128	462304	29.1***
	Cultures	152	2415589	15892	
	Total	184	7063017	.....	
B	Densities	10	5270956	527096	51.2***
	Cultures	174	1792061	10299	
	Total	15	229184	.....	
	Densities	7	222673	31810	55.0***
C	Males	1	2465	2465	4.3
	Residual	7	4046	578	
	Total	15	209675	.....	
	Densities	7	206507	29501	129.2***
D	Males	1	1569	1569	6.9*
	Residual	7	1599	228	

To convert mean squares to decimal equivalents multiply each MS by 10^{-7}.

Since logarithmic transformations made no difference in any of the results, all of the analyses are based on untransformed data. For parts A and B of the table it was not practical to adjust for strain difference and not necessary to adjust for heterosis since only frequencies of ebony were involved. So analyses are based on frequencies of ebony calculated directly from raw data. In both the ♀×(♂) and ♀×♂ sets of crosses the effect of crowding is significant, with $P < 0.001$ for densities, although comparison of the mean squares indicates considerable variation among cultures. Comparison of the two sets, ♀×(♂) and ♀×♂, again reveals density to be the more significant factor; but in the unadjusted data (part C) the effect of males is not significant; while adjustment of the data for strain difference

(part D) discloses that the males do produce a significant effect, with $P < 0.05$. Thus an increase in fecundity and a decrease in the frequency of mutant offspring are both associated with the continued presence of males. A similar relation between fecundity and number of mates has been reported by BATEMAN (1948) for flies from mass cultures.

The viability coefficients of Table 3 are based on the following considerations.

α', β', γ', = theoretical frequencies of $+/+$, $+/e$, e/e, respectively.

α, β, γ, = experimental frequencies of $+/+$, $+/e$, e/e, respectively.

V = the total coefficient of relative viability from egg to adult (including egg fertility). Then, computed as components of selection coefficients, the viabilities for $+/+$, $+/e$ and e/e are, in order, $\alpha\beta'/\beta\alpha'$, 1, and $\gamma\beta'/\beta\gamma'$. If theoretical frequencies for $+/+$, $+/e$ and e/e are as 1:2:1, then the viability coefficients become $2\alpha/\beta$, 1, and $2\gamma/\beta$, respectively. Since all viabilities in this paper are calculated from pooled data rather than by averaging the viabilities of individual cultures, no adjustment for bias is made (HALDANE 1956; STERN *et al.* 1952).

V_1 = the genetic component of V, i.e., that independent of crowding.

$\hat{V}$ = the environmental component of V, i.e., that dependent upon crowding (other environmental factors are regarded, in theory, as being constant from culture to culture).

v, v_1 ($= \dot{v}$ of MOREE 1952), and $\hat{v}$ = the coefficients of inviability; they are complements of V, V_1 and $\hat{V}$, respectively, such that $V + v = V_1 + v_1 = \hat{V} + \hat{v} = 1$.

Then these relations and their limits follow:

$$V = V_1\hat{V}, \quad v = v_1 + \hat{v}V_1, \quad \hat{V} = V/V_1, \quad \text{and } \hat{v} = (v - v_1)/(1 - v_1);$$
$$\lim V = V_1 \text{ as } \hat{V} \to 1, \quad \lim v = v_1 \text{ as } \hat{v} \to 0, \quad \lim \hat{V} = 1 \text{ as } V \to V_1, \quad \text{and } \lim \hat{v} = 0 \text{ as } v \to v_1$$

The limiting condition can be approximated experimentally if the crowding resulting from population density can be made to approach a minimum, as it does in the cross $1 \times (1)$. Thus a reasonably good estimate of $\hat{V} = V/V_1$ can be made for any level of crowding by assuming that under minimal crowding $V = V_1$ and that under all degrees of crowding V_1 is constant. $\hat{V}$ decreases, of course, as crowding increases. The ratio $\hat{v}/v$ taken at different degrees of crowding shows that as crowding increases $\hat{v}$ becomes a major component of v. Thus the decrease in the relative viability (V) of the mutant is accounted for by the increase in the environmental component of inviability ($\hat{v}$).

The designation of V_1 as the genetic component of viability perhaps requires clarification. In the $1 \times (1)$ cross the deviation of the frequency of e/e progeny from 25 percent is small and, for this sample size, is not statistically significant. But it is considered as having biological significance for two reasons. (1) As crowding increases, the frequency of e/e decreases. This indicates a potential difference in viability between the carriers of $+/-$ and e/e, even though such a difference might not be apparent under minimal crowding. (2) The deviation acquires statistical significance when sample size is increased by combining the

data of the first four ♀×(♂) and the first two ♀×♂ crosses. The pooled data give the following results: frequency of $e/e = 23.35$ percent; viability of $e/e = 88.97$ percent; for an expected 3:1 ratio, $x^2 = 8.1$, and P for one degree of freedom is between 0.05 and 0.01; for the heterogeneity test, calculated $x^2 = 1.0$. The small deviation does, therefore, reflect a real viability difference. The pooled data might be used to estimate the condition $V=V_1$ and to compute $\hat{V}$. But since all sample sizes would probably reflect significance if they could be sufficiently increased, and in consideration of the x^2 values for the lower degrees of crowding in Table 1, it seems preferable to use the results of the 1×(1) crosses to estimate the $V=V_1$ relation. A corresponding condition has been discussed by LEWONTIN (1955) and designated by SANG (1949) as the "minimal mortality."

In a general review, SANG (1950) has indicated that the fecundity of flies in population bottles is reduced by (1) food shortage during their larval life and by (2) changes in the character of the yeasts that grow in the medium on which these flies oviposit. The latter condition, according to SANG, may more than halve the potential fecundity. He also indicates that in population bottles fecundity is reduced very little if at all by adult crowding *per se*, by larval interference with feeding and oviposition, by competition for oviposition sites, or by adult food shortage (provided larval nutrition has been adequate). Flies used in the experiments reported in this paper were certainly subject to SANG's second condition and possibly to his first, for since in all stock cultures flies were given food ample for the production of large numbers of offspring, such cultures were nevertheless maintained by mass transfer of large numbers of adult flies. This suggests (1) that any reduction of fecundity as may have occurred in the density series is comparable to that occurring in cage populations and (2) that inviability at or near the 200 × 200 level of the density series (that at which each female produces an average of about 2.5 offspring) is fairly near to that occurring in numerically stable cage populations. Compared with cage populations, levels above 200 × 200 in the density series probably represent "super-crowding." Further, Table 3 shows that in the ♀×♂ set of crosses the total number of offspring for a given cross is higher, and the proportion of mutant progeny is lower than in the corresponding cross of the ♀×(♂) set, a relation expected to be just the reverse if mere crowding of adults were to lower fecundity. This is in agreement with findings reviewed by SANG (1950) and reported by BATEMAN (1948). The possible implication that viability comprises the major part of adaptive value involves, in the present case, a situation too complex to prejudge.

It is well known that preadult life is prolonged under conditions of stringency, such as those resulting from crowding. The present experiments provide no data of the sort necessary for directly determining how such prolongation affects the outcome of a cross. Several pieces of information, however, are suggestive. The average time between introduction of nonvirgin parents into a culture bottle and emergence of the first offspring ranged from 10.3 days in the least crowded cultures to 13.0 days in the most crowded. Since an eight day counting period followed the date of first emergence, the "life" of these cultures ranged from 18 to 21 days. The data reviewed by SANG (1949, 1950) show that (1) preadult

crowding of wild-type flies prolongs preadult life by an average of about 50 percent, that (2) in an extreme case larvae living for as long as 14 days formed pupae that were "only rarely viable," that (3) in population bottles about 82 percent of all eggs are laid during the first four days of a seven day period of ovipositing, and about 92 percent during the first five, and that in general (4) percentage mortality and length of preadult life are proportional. Combining all of this, we see that eggs laid on the day that parents are removed from a 1600 × 1600 cross have a 14 day period within which to develop and emerge as adults, if they do emerge, and that probably only a small percentage of all eggs laid are so involved. Although comparative data on +/− and *e/e* flies are lacking with respect to this point, it nevertheless seems reasonable to suppose that loss due to a prolonged preadult period does not seriously affect the outcome of the present experiments.

The results of these experiments indicate that the relative viability of *e/e* is reduced under conditions of crowding, and that the increase in crowding accounts, in general, for a larger and finally for a major proportion of the total inviability. A question then arises as to whether the environmental portion of inviability results from crowding among the larvae themselves or from an effect of parental crowding upon the larvae. Part D of Table 4 shows that the parental males are associated with the difference between the results of the ♀×(♂) and ♀×♂ crosses, but they might account for the lower viabilities of the ♀×♂ crosses either directly (adult effect), or by causing denser progeny populations to be produced (larval effect), or both. A similar question can be raised with respect to the ♀×(♂) set, considered by itself. The experiments that follow have been devised to test these and certain related possibilities. Since all flies used in these next experiments were from new strains, adjustment for strain difference has not been necessary.

Variable crowding of adults: The plan of this experiment was such that parental adult crowding, in terms of adult fly days, varied by a factor of about 150, while larval crowding was held relatively constant. Heterozygous females and males were placed together for a two-day fertilization period. Following this, five flies of each sex were placed in each of 12 half-pint culture bottles for a two-day egg laying period and then removed. Additions of adult flies were then made as follows: in one set of bottles, 0 flies; in a second set, 200 virgin females; in a third, 200 males; and in a fourth, 600 males. Each bottle contained 50 ml of medium and an application of live yeast suspension, 0 ml before introduction of the parental flies, one drop at the time of their removal two days later, and one ml at the time of removal of the added adults five days later still. Other conditions and methods were as already described. Each set was repeated three times to give the data of Table 5. According to the analysis of variance the addition of adult flies causes no significant variation among the sets, while the x^2 test for the pooled data indicates a slight but significant *excess* of the mutants. Why these conditions reduce the relative viability of the wild type is unexplained; possibly the mutants have a shorter emergence time, but this seems unlikely, especially since KALMUS (1945) reported just the opposite for the mutant strain

TABLE 5

Effect of different degrees of adult crowding on the frequency of ebony progeny from the cross 5 +/e × 5 +/e

Adult flies added	Adult fly-days	Number counted			Percent e/e	χ^2	
		+/−	e/e	Total		(3:1)	(h)
0	20	819	286	1105	25.88	0.5	3.9
200 ♀ ♀	1020	905	322	1227	26.24	1.0	1.0
200 ♂ ♂	1020	771	289	1060	27.26	2.9	0.1
600 ♂ ♂	3020	786	291	1077	27.02	2.3	0.5
Total		3281	1188	4469	26.59	6.0*	6.3

Analysis of variance of the frequencies of ebony

Source	DF	SS	MS	F
Total	11	32837	.....	....
Adult densities	3	5968	1989	....
Cultures	8	26869	3359	....

h = heterogeneity. To convert mean squares to decimal equivalents multiply each MS by 10^{-8}.

that he used. But at least, as is evident in this instance, the adult population produces no selective effect against the second generation mutants.

Variable crowding of progeny: In this experiment the variables were reversed relative to the test just described. Following a four-day fertilization period +/*e* flies were placed, 200 of each sex in each of four bottles, and in a second set, 400 of each sex in each of four bottles. After seven days the parents were removed and a quadrant of medium with its contained eggs and larvae was taken from each culture, divided into four parts, and each part transferred to a fresh bottle and given abundant food. All cultures were of pint size and contained 80 ml of medium. Each culture received one drop of live yeast suspension before introduction of the parents and each transfer culture received in addition five ml of suspension at the time of transfer. Thus the volume ratio of suspension per 80 ml of medium was approximately 1:50:1400 for the nontransfer bottles, density series bottles, and transfer bottles, respectively.

The results of this experiment are summarized in Table 6. The difference between frequencies of *e/e* progeny from transferred and from untransferred larvae is large and, as shown by the analysis of variance, significant. The two heterogeneity χ^2 values associated with the transferred larvae were introduced by a single culture; though significant, they are not important in the present case. In transfer cultures the increase in *frequency* of *e/e* progeny is fivefold, compared with that of the nontransfers. If the observed numbers of progeny from the transfer cultures are multiplied by three (since a quadrant of medium was transferred from each original culture), then it is seen that the increase in *number* of *e/e* progeny is fifty-six fold.

Even if parental infecundity were introduced by crowding, it would have no bearing, in this case, on the relation between the transfer and nontransfer cultures. But since the transferred pieces of medium were placed in fresh cultures containing a whole cake of medium, there is a question as to whether the high

TABLE 6

Effect of larval transfer and extra food on the numbers and frequencies of ebony progeny from the cross +/e × +/e, under two different degrees of adult crowding

Cross	Number counted			Percent	χ^2	
	+/−	*e/e*	Total	*e/e*	(3:1)	(h)
			Larvae not transferred			
200 × 200	1285	62	1347	4.60	298.9***	1.4
400 × 400	1482	64	1546	4.14	358.8***	0.2
Total	2767	126	2893	4.36	657.6***	1.7
			Larvae transferred			
200 × 200	3580	1129	4709	23.98	2.6	13.3**
400 × 400	4666	1240	5906	21.00	50.5***	1.8
Total	8246	2369	10615	22.32	40.7***	27.6***

Analysis of variance of logarithms of the frequencies of ebony

Source	DF	SS	MS	F
Total	15	2.2669	.....	
Parental densities	1	0.0045	0.0045	
Larval transfers	1	2.1389	2.1389	225.2***
Interaction	1	0.0039	0.0039	
	13	0.1235	0.0095	
Error	12	0.1196	0.0100	

h = heterogeneity.

frequency of transferred *e/e* progeny resulted from their having had extra food or extra space, since they had both. An idea of this can be got by comparing the *e/e* frequencies of the nontransfers with the *e/e* frequencies of the same degrees of parental crowding in the density series. Those of the nontransfers are much the lower, and since in these the larvae had the same space, but only about two percent of the quantity of yeast suspension as the larvae of the density series, it is suggested that food, not space, is the effective factor. The extra food, then, results in the higher survival rate of the *e/e* offspring and the opposite condition, insufficiency of food, would account for their lowered frequency in crowded cultures.

Variable crowding of both adults and progeny: Following a two-day fertilization period, fertilized and virgin +/*e* females in varying proportions were placed in half-pint culture bottles for the usual seven day period. Each culture contained 50 ml of medium with added live yeast suspension, one drop before introduction, of the adult females and one ml at the time of their removal. Other procedures and conditions did not differ from those already described. Four sets of three bottles each provided the data of Table 7. It is clear that sets 1 and 2, with low crowding of fertile females, give a significantly higher frequency of *e/e* progeny than do sets 3 and 4, in which the degree of crowding of the fertile females is higher. Variation in number of virgin females has little relation to the outcome of the experiment, nor does variation in number of adult fly days. It is not surprising that sets 3 and 4 have lower *e/e* frequencies than do the closest corre-

TABLE 7

Effect of different degrees of larval and adult crowding on the frequency of ebony progeny from the cross +/e × +/e

Set	f♀♀ + v♀♀	Adult fly-days	Number counted +/-	Number counted e/e	Number counted Total	Percent e/e	χ^2 (3:1)	χ^2 (h)
(1)	1 + 1	14	803	257	1060	24.25	0.3	1.4
(2)	1 + 199	1400	385	121	506	23.91	0.3	2.5
(3)	199 + 1	1400	1527	124	1651	7.51	269.3***	5.7
(4)	199 + 199	2786	2310	216	2526	8.55	364.5***	8.1*
	Total		5025	718	5743	12.50	478.4***	174.1***

Analysis of variance of logarithms of the frequencies of ebony

Source	DF	SS	MS	F
Total	11	0.8758	.....	
Fertile female densities	1	0.7500	0.7500	54.4***
Virgin female densities	1	0.0017	0.0017	
Interaction	1	0.0085	0.0085	
	9	0.1241	0.0138	
Error	8	0.1156	0.0145	

h = heterogeneity; f = fertile; v = virgin.

sponding levels of parental crowding in the density series, the latter having had about twice the amount of yeast suspension per unit of surface area of medium. Two peculiarities, the low number of progeny in set 2 (due to pupation in the medium) and the significant heterogeneity of χ^2 for set 4, do not invalidate the general result, which, as is shown by the analysis of variance strongly corroborates the relation between high larval density and low frequency of *e/e* progeny found in the preceding experiments.

Components of viability and inviability: It is interesting to attempt an additional use of the first three sets of data of Table 7 to divide coefficients of viability and inviability into components related to genetic, larval and adult effects, as is shown in Table 8. These components have the following definitions and relations.

V_2 = the larval effect, or larval density component of $\widehat{V}$, i.e., that dependent upon crowding among the larvae themselves.

V_3 = the adult effect, or adult density component of $\widehat{V}$, i.e., that dependent upon crowding among the parents or among the adults of a parental generation.

v_2 and v_3 = the coefficients of inviability, complementary to V_2 and V_3, such that $V + v = V_1 + v_1 = V_2 + v_2 = V_3 + v_3 = 1$.

Then

$$V = V_1\widehat{V} = V_1V_2V_3 = (1-v_1)(1-v_2)(1-v_3). \qquad (1)$$

The densities of the virgin females and of the fertile females and their progeny, in the first three sets of Table 7, makes possible the following further relations, used for estimating viabilities V_1, V_2 and V_3 of Table 8.

TABLE 8

Effect of different degrees of larval and adult crowding on viability and inviability and their components, with respect to e/e

Set	f♀♀ + v♀♀	Frequencies (percent) +/+ (α)	+/e (β)	e/e (γ)	Viabilities (percent) +/+ ($2\alpha/\beta$)	e/e ($2\gamma/\beta$)
(1)	1 + 1	23.87	51.88	24.25	$92.04 = V$	$93.47 = V_1$
(2)	1 + 199	23.98	52.11	23.91	$92.04 = V$	$91.78 = V_{13}$
(3)	199 + 1	29.15	63.34	7.51	$92.04 = V$	$23.72 = V_{123}$

Components	Viabilities	Inviabilities	Proportional inviabilities Order 1	Order 2
Genetic	$V_1 = 93.47$	$v_1 = 6.53$	$[v_1] = 8.56$	$[v_1] = 8.56$
Larval	$V_2 = 25.84$	$v_2 = 74.16$	$[v_2] = 90.87$	$[v_2] = 89.26$
Adult	$V_3 = 98.19$	$v_3 = 1.81$	$[v_3] = 0.57$	$[v_3] = 2.18$
Total	$V = 23.72$	$v = 76.28$	$[v] = 100.00$	$[v] = 100.00$
Genetic	$V_1 = 93.47$	$v_1 = 6.53$	$[v_1] = 8.56$	$[v_1] = 8.56$
Environmental	$\hat{V} = 25.37$	$\hat{v} = 74.63$	$[\hat{v}] = 91.44$	$[\hat{v}] = 91.44$
Total	$V = 32.72$	$v = 76.28$	$[v] = 100.00$	$[v] = 100.00$

Frequencies of ebony progeny are from Table 7. Genotype frequencies are adjusted for heterosis as in Table 3.

Set (1) $v_1 = k, v_2 \to 0, v_3 \to 0$; so $\lim V = V_1$.
Set (2) $v_1 = k, v_2 \to 0, v_3 \to \max$; so $\lim V = V_{13} = V_1V_3$, and $V_3 = V_{13}/V_1$.
Set (3) $v_1 = k, v_2 \to \max, v_3 \to \max$; so $\lim V = V_{123} = V_1V_2V_3$, and $V_2 = V_{123}/V_{13}$.

To get proportional components $[v_1]$, $[v_2]$ and $[v_3]$ of inviability such that their sum equals unity, requires consideration of the biological order in which the genetic, larval and adult effects occur, and of the possibility that some may occur simultaneously. Thus the three effects may be ordered in 13 different ways when all possible combinations and arrangements are taken into account. Only two orders are considered here. Suppose the three effects occur during preadult life in the order v_1, v_2 and v_3. Then from (1), $v = v_1 + v_2V_1 + v_3V_2V_1$, and dividing each term in this expression by v, gives

$$v_1/v + v_2V_1/v + v_3V_2V_1/v = [v_1]+[v_2]+[v_3] = 1. \qquad \text{(order 1)}$$

On the other hand suppose that the larval and adult effects occur simultaneously, following the genetic effect, in which case the proportional components will have different values. Then from (1), $v = v_1 + \hat{v}V_1$, and dividing each term in this expression by v gives $v_1/v + \hat{v}V_1/v = [v_1] + [\hat{v}] = 1$; applying proportionality factors to $[\hat{v}]$ gives the required expression as

$$v_1/v + v_2[\hat{v}]/(v_2 + v_3) + v_3[\hat{v}]/(v_2 + v_3) = [v_1] + [v_2] + [v_3] = 1. \text{(order 2)}$$

The application of these expressions to the data of Table 7, summarized in Table 8, shows a striking difference in the magnitudes of the larval and adult components, with both orders. A comparison of these effects, larval/adult, is interesting. An attempt made by taking the ratio of simple effects, as $(b-(1))/(a-(1)) = (V_{123}-V_1)/(V_{13}-V_1)$, leads to $\hat{v}/v_3$ and does not give the compari-

son desired. On the other hand the proportional inviabilities can be used directly, taking $[v_2]/[v_3]$. For the first order this gives $90.9/0.6 = 151.5$. For the second, $89.3/2.2 = 40.6$; in this case $[v_2]/[v_3]$ reduces to v_2/v_3 and of course gives the same result if the numbers are not too rounded off. The second order seems the more plausible but the first is in better agreement with the results of the variance analysis, although variance analysis and viability components do not, we realize, measure quite the same thing.

Regardless of their size or statistical significance, the larval and adult components may be combined to obtain a general environmental component. Since in this case the adult component is both small and statistically nonsignificant, the combination should be made (Table 8), noting that $\hat{V} = V_2V_3$, that $\hat{v} = v_2+v_3-v_2v_3$, and that $[\hat{v}] = [v_2] + [v_3]$. Using proportional inviabilities, the genetic and environmental components can be compared directly. The relation between the two is, of course, apparent and justifies the assumptions made in calculating the environmental component ($\hat{V}$) in the section on the density series.

DISCUSSION

For the materials and methods used, the experiments reported in this paper show that the three genotypes studied fall into the following sequence with respect to relative viability: $+/e^{11} > +/+ > e^{11}/e^{11}$. Crowding of the larvae, with consequent food shortage, does not affect the relative viabilities of $+/+$ and $+/e^{11}$, but it does lower that of e^{11}/e^{11}. Although the greatest numbers of progeny, including mutants, are produced at the 25-female level of the density series, the highest relative viability of e^{11}/e^{11} occurs when crowding is at a minimum and the lowest when it is at a maximum. A part of the inviability of e^{11}/e^{11}, which occurs even when crowding is minimal, is interpreted as being independent of crowding and is therefore regarded as genetic. Another part, which increases with crowding, is regarded as environmental. This part in turn is divisible into a larval component, which varies with crowding, and an adult component, which is negligible. Summarizing symbolically: v_1 is small and fairly constant, v_2 is in general proportional to crowding, and v_3 is negligible.

The differential viability of coexisting genotypes may occur even when crowding is kept to a minimum (STERN, *et al.* 1952; KERR and WRIGHT 1954; SPOFFORD 1956) and the relative viability of the mutant may be considerably lowered when larval crowding is increased. The latter relation has been observed when crowding occurs as a consequence of heavy oviposition (HARNLY 1929; MOREE 1952; LEWIS 1954; MOREE, NEWHOUSE and KING 1957) and when it results from rearing large, known numbers of larvae transferred without the parents to separate cultures (LEWONTIN 1955). Absolute viability may also be lowered when the density of transferred larvae is sufficiently increased in cultures of single strains. This has been demonstrated by LEWONTIN (1955) for a number of different wild-type strains and for one of white eye (w), and by SANG (1949) for his wild-type strain, the inviability in this case having also been demonstrated as largely due to food shortage.

DOBZHANSKY (1947) and WALLACE (1948), working with various karyotypes of *D. pseudoobscura*, and DACUNHA (1949) with body-color genotypes of *D. polymorpha*, found that the differential viability of the several types was reduced when larval populations were uncrowded with respect to food supply. BIRCH (1955) found further that crowding of adults, rather than of larvae, had no evident effect on selection and ostensibly none on the viability of karyotypes of several of the most studied gene arrangements of *D. pseudoobscura*. That, in general, homozygotes not only have a lower viability than heterozygotes but are also more sensitive to environmental differences (as is e^{11}/e^{11} in the present study) has been shown by DOBZHANSKY and LEVENE (1955) and is extensively discussed by them. On the other hand SPIESS (1958), using a density series of transferred eggs, observed that crowding did not alter the relative viabilities of several karyotypes of *D. persimilis*, within the limits of his tests. Other instances of an independent relation between viability and density have been noted by DOBZHANSKY and SPASSKY (1944) and LEWONTIN (1955); these authors also noted that some genotypes are most viable at high, others at intermediate, and still others at low densities.

With respect to viability, the superiority of a heterozygous genotype over both of its corresponding homozygous types, though not invariable, has been observed so frequently as to require little comment (DOBZHANSKY 1947; WALLACE 1948; DACUNHA 1949; STERN *et al.* 1952; KERR and WRIGHT 1954; LEWIS 1954; DOBZHANSKY and LEVENE 1955; SPIESS 1958). Theoretically, the percentage of heterozygous loci in the near vicinity of e^{11} would be expected to be greater in the $+/e^{11}$ flies than in either of the homozygotes; but this is only inferential and, in addition, heterosis may be due to more than heterozygosis *per se*. Whether the differential viabilities of the carriers of $+/+$, $+/e^{11}$ and e^{11}/e^{11} are due to single gene effects (MUKAI and BURDICK 1959), or to the differential effects of these in conjunction with the effects of other genes in the genetic background (STERN *et al.* 1952), or to the action of an extreme mutant that overrides the functions of other genes (BUZZATI-TRAVERSO 1955), are possibilities that cannot readily be distinguished in the present data. The viability of a given genotype is demonstrably a function of many variables: its own properties, its genetic background with respect to both individuals and populations of individuals, its state of nutrition, and the many aspects of its physical environment (WALLACE and KING 1952; DOBZHANSKY and LEVENE 1955; LEWONTIN 1955). Hence in the present case the genotypes of e^{11} and its wild-type allele may perhaps be considered as the "affected" as well as the "effecters" with respect to the total genetic effect on viability.

The results cited from other authors, while providing confirmation of those of the present study, also place some limitations on their generality. For, differences of strain, genotype and experimentation, especially with respect to the extent to which a particular factor is tested, produce results some of which are quite unpredictable and variations which are not always easy to compare. Thus the results of the present study do not conform with, but are not necessarily in conflict with those of REED and REED (1950) and MERRELL and UNDERHILL (1956), who made

no extensive density tests but, for other reasons, considered that larval inviability was not important as a selection component in their cage populations.

L'HÉRITIER and TESSIER (1937) and later KALMUS (1945), TESSIER (1947) and RASMUSSEN (1958) hypothesized the superior fitness of heterozygous over both homozygous genotypes as explaining the persistence of the gene *e* in their experimental populations. CARSON (1958) came to a similar conclusion for the gene e^s. The results of the present experiments demonstrate the superior viability of $+/e^{11}$ and thus verify the hypothesis as far as the viability component of fitness is concerned. They also show that inviability is an effective component of selection against e^{11}. If we assume, momentarily, that inviability comprises the whole of selection, that it is constant, and that mating is random, a provisional equilibrium frequency can be determined for e^{11}. In a cage population with crowding equivalent to that of the 200×200 level of the density series this would be calculated as $0.08/(0.08 + 0.52) = 0.13$, a value in the neighborhood of those reported by L'HÉRITIER and TESSIER for several of their populations, but a bit lower than the lowest of those deducible from KALMUS' data. A more complete comparison must await the evaluation of other components of selection.

SUMMARY

1. Genotypes segregating from the original cross of wild type $\times$ recessive ebony[11] of *Drosophila melanogaster* were tested for their response to variable crowding. Variable crowding was accomplished by a density series ranging from one fertilized F_1 female to 1600 F_1 flies of each sex *per* culture.

2. At all stages of crowding the relative viabilities of the F_2 genotypes formed the sequence: $+/e^{11} > +/+ > e^{11}/e^{11}$.

3. Increased crowding of parental adults, and hence of larvae, did not affect the relative viabilities of $+/+$ and $+/e^{11}$, but it consistently lowered that of e^{11}/e^{11}. From the lowest degree of crowding to the highest, the frequency of e^{11}/e^{11} ranged from 23.9 to 7.9 percent; viability ranged from 91.5 to 25.1 percent. The highest mutant viability, therefore, coincided with the least and the lowest mutant viability with the highest of the degrees of crowding; but the greatest *numbers* of offspring, including mutants, were produced at an intermediate (25-female) level of crowding.

4. The presence of parental males was associated with a slight increase in female fecundity and a slight decrease in frequency of mutant offspring.

5. Possible complications resulting from infecundity and prolonged emergence time are discussed.

6. Some inviability occurred even when crowding was minimal. Experiments involving variation of adult crowding, preadult crowding, and quantity of food disclosed that adult crowding had little if any effect on the inviability of mutant progeny; larval crowding, with resulting food shortage, caused most of the inviability and the larval effect increased with increased crowding.

7. Mutant inviability was divided into three components: (a) a small, fairly constant component appearing under minimal crowding, regarded as genetic and estimated at about 9 percent; (b) a larval component resulting from food

shortage among crowded larvae, generally proportional to crowding and estimated at a maximum of about 90 percent; (d) a component associated with adult crowding, estimated at from 0.5 to 2.0 percent, but found to be nonsignificant, statistically.

8. Heterosis, identification of the determining genetic elements, and causes of variation relating to comparison of results, are discussed briefly.

9. Relations of the present study to population cage experiments on the alleles of ebony are also discussed.

ACKNOWLEDGMENT

We would like to express our appreciation to PROFESSORS TH. DOBZHANSKY and JOHN W. GOWEN for discussion and comments given at several times during the course of work. Our thanks are also due to DR. THOMAS S. RUSSELL, Washington Agricultural Experiment Stations, for examining the statistical work and for several suggestions on the variance analyses, and to DR. VERNE F. NEWHOUSE, and others, for assistance in the laboratory.

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ACKNOWLEDGMENT

The Editors wish to express their appreciation to the Genetics Society of America for a gift which has made it possible to increase the size of the current volume so as to include manuscripts whose publication otherwise would have had to be deferred or declined.

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GENETICS

A PERIODICAL RECORD OF INVESTIGATIONS
BEARING ON HEREDITY AND VARIATION

Founded in 1916
by
GEORGE H. SHULL
with the cooperation of

WILLIAM E. CASTLE
EDWIN G. CONKLIN
CHARLES B. DAVENPORT
BRADLEY M. DAVIS
EDWARD M. EAST
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VOLUME 46—1961

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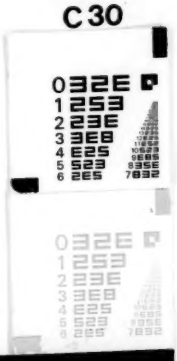
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